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**Characterisation of the agent strain in
sporadic and variant Creutzfeldt-Jakob
disease by transmission to wild-type mice**

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**Doctor of Philosophy
University of Edinburgh
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Declaration

I declare that this thesis has been composed entirely by myself and that the work presented herein is my own, except where otherwise stated. No part of this thesis has, or will be, submitted for any other degree, diploma or qualification.

This thesis involves the investigation of mouse transmission experiments carried out between 1995 and 2002. I was not directly involved in either the planning or the animal husbandry aspect of the transmission experiments. However, I declare that I have carried out all the laboratory tests on collected fixed and frozen tissue and performed all of the data analysis presented, unless otherwise stated.

Diane L Ritchie

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Abstract

Transmissible spongiform encephalopathy (TSE) strains are defined by their biological properties on transmission to wild-type mice, specifically by their characteristic incubation periods and patterns of vacuolar pathology ('lesion profiles') in the brain. Whilst a single TSE strain has been identified in variant Creutzfeldt-Jakob disease (vCJD), the phenotypic heterogeneity observed in sporadic CJD (sCJD) implies the existence of multiple strains of agent. These distinct strains are proposed to be enciphered by the different conformers of abnormal prion protein (PrP), recognised as different protease resistant PrP (PrP^{res}) types by Western blotting (type 1 or type 2) and are thought to be substantially influenced by the different prion protein gene (*PRNP*) codon 129 polymorphism (MM, MV and VV).

To test the relationship between disease phenotype and agent strain, this study carried out a full characterisation of the sCJD agent by primary transmission of brain tissue from 27 sCJD cases (comprising all six possible combinations of *PRNP* codon 129 genotype and PrP^{res} type) in panels of wild-type mice using the standard strain typing properties of incubation period and lesion profiles, plus a full analysis of PrP in the mouse brain and the PrP^{res} molecular subtypes present. Results were directly compared with the transmission characteristics of brain tissue from 10 vCJD cases. The characterisation of the agent strain in sCJD and vCJD was extended to include analysis of subsequent mouse-to-mouse passages. In an additional investigation, wild-type mice were experimentally challenged with a wide-range of lymphoid tissues, neural tissues and biological fluids from vCJD and sCJD patients in order to investigate the extent of peripheral involvement in CJD and to determine whether the agent is subject to any tissue-specific modifications.

Analysis of all 27 sCJD sources demonstrated the existence of two strains of agent, one associated with the MM1/MV1 subgroups and the other associated with the MM2 subgroup, which could be distinguished by their transmission properties in the mice. The lack of transmission in mice challenged with VV1, MV2 and VV2 tissues provided evidence of at least one further sCJD strain. In contrast, all 10 vCJD

sources resulted in consistent incubation periods and lesion profiles, suggesting that all 10 patients investigated were infected with the same strain of agent. Overall, the observation that PrP^{res} type in sCJD and vCJD was maintained on transmission is consistent with the proposition that PrP^{res} type plays a role in enciphering strain-specific information. Experimental transmissions from peripheral tissues extended the evidence for a peripheral infection in vCJD. However, comparison of incubation periods and lesion profiles from transmission of brain and peripheral tissues showed no evidence of tissue-specific modification in the biological properties of the agent. Furthermore, the detection of low levels of infectivity in a sCJD buffy coat sample provides supporting evidence for a peripheral involvement in sCJD.

This study highlights the complex relationship between disease phenotype, *PRNP* codon 129 genotype, PrP^{res} type and agent strain in sCJD and vCJD. Overall, this study confirms that multiple strains of agent are associated with sCJD, some of which successfully propagate in wild-type mice but none of which are identical to the agent responsible for vCJD. Importantly, the sCJD strains identified here by their biological properties partially correlated with the current sub-classification system for sCJD which is based on the clinical and pathological phenotype of the disease.

Abbreviations

ArcH	arcuate hypothalamic nucleus of the hypothalamus
BSE	bovine spongiform encephalopathy
BP	brain pathology number
CJD	Creutzfeldt-Jakob disease
CDI	conformational-dependant immunoassay
CNS	central nervous system
CSA	catalysed signal amplification system
CSF	cerebrospinal fluid
CWD	chronic wasting disease
C57BL x VM	FI cross of inbred wild-type C57BL and VM mouse lines, (murine <i>Prn-p^{ab}</i>)
C57BL	inbred wild-type C57BL/FaBtDk mouse line (murine <i>Prn-p^a</i>)
dpi	days post injection
dLGN	dorsal lateral geniculate nucleus of the thalamus
DRG	dorsal root ganglia
fCJD	familial Creutzfeldt-Jakob disease
FFI	fatal familial insomnia
FSE	feline spongiform encephalopathy
GFAP	glial fibrillary acidic protein
GPI	glycosyl-phosphatidylinositol anchor
GSS	Gerstmann-Sträusler-Scheinker disease
GT	gene targeting
H&E	haematoxylin and eosin staining. A common histological stain demonstrating cellular architecture of tissue
hGH	human growth hormone
HPA	Health Protection Agency
IC	intracerebral inoculation
iCJD	iatrogenic Creutzfeldt-Jakob disease
ID	infectious doses
IHC	immunohistochemistry
IP	intraperitoneal inoculation
IVC	individual ventilated cases
JGV	Journal of General Virology
kDa	(kilo)Dalton
LREC	Lothian Research Ethics Committee
MHb	medial habenular nucleus of the thalamus
MM	methionine homozygous at codon 129
MV	methionine/valine heterozygote at codon 129
NCJDRSU	National CJD Research & Surveillance Unit
NPD	Neuropathogenesis Division, Roslin Institute
NPU	Neuropathogenesis Unit, Institute for animal health
PAGE	polyacrylamide gel electrophoresis
PK	proteinase K
PMCA	protein misfolding cyclic amplification
PrP	prion protein

PrP ^C	PrP cellular, non-infectious form
PrP ^d	disease-associated form of PrP
PrP ^{res}	proteinase K resistant form of PrP
PrP ^{Sc}	PrP scrapie: disease-associated form of PrP
PrP ^{sen}	proteinase K sensitive form of PrP
<i>PRNP</i>	PrP gene encoding human PrP
<i>Prn-p</i>	PrP gene encoding mouse PrP
<i>Prn-p^a</i>	murine PrP 108L and 189T
<i>Prn-p^b</i>	murine PrP 108F and 189 V
PSPr	protease-sensitive prionopathy
RGI	random genetic insertion
RU	laboratory accession number
RIII	inbred wild-type RIII/FaDK mouse line (murine <i>Prn-p^a</i>)
sCJD	sporadic Creutzfeldt-Jakob disease
SDS	sodium dodecyl sulphate
TBS	tris buffered saline
TME	transmissible mink encephalopathy
TRG	trigeminal ganglia
TSE	transmissible spongiform encephalopathy
VPSPr	variably protease-sensitive prionopathy
vCJD	variant Creutzfeldt-Jakob disease
VM	inbred wild-type VM/Dk mouse line (murine <i>Prn-p^b</i>)
VV	valine homozygous at codon 129

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Chapter 1: Transmissible spongiform encephalopathies

1.1 General Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of rare degenerative and invariably fatal diseases affecting the central nervous system (CNS), which can occur in humans as well as a number of animal species. Scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in mule deer and elk and Creutzfeldt-Jakob disease (CJD) in humans are just some of the more familiar members of this group of diseases (Table 1.1). These disorders are defined as ‘transmissible’, referring to the ability of infected tissue to induce disease following inoculation into or ingestion by subjects from the same species and in some instances individuals of a different species. Recent reports of five probable cases of transmission of variant CJD (vCJD) infectivity through transfusion with blood and blood products from asymptomatic donors who went on to develop vCJD, is a clear example of TSE transmission within a single species (Llewelyn et al., 2004; Peden et al., 2004; Wroe et al., 2006; Health Protection Agency, 2009a; Peden et al., 2010). Furthermore, the now convincing evidence supporting the initial hypothesis that vCJD is caused by human infection with the BSE agent is a recent demonstration of cross-species transmission between cattle and human (Collinge et al., 1996; Lasmezas et al., 1996; Bruce et al., 1997; Hill et al., 1997; Scott et al., 1999).

The term ‘spongiform’ derives from the characteristic lesions or vacuoles that occur within the neuropil of the brain of an affected subject (human or animal). Vacuoles can vary in size from 2-20 microns and in some TSEs become confluent, resulting in irregular cavities within the neuropil. Examination of spongiform change has shown that the majority of vacuoles occur within neuronal processes (mainly neurites) and cell bodies, depending on strain of TSE, and ultrastructural studies suggest that they can originate in several sub-cellular organelles or processes (Jeffrey et al., 1995). In experimental models of TSEs, vacuolation has been shown to vary between the different TSE sources and the host species (Fraser and Dickinson, 1968). Although not absolutely specific for TSEs, the appearance of vacuolar pathology is of

considerable diagnostic importance. The term encephalopathy is given to these diseases as the majority of the pathological changes are associated with the brain.

In humans, TSEs are unique in that they can have an idiopathic (sporadic), familial (genetic) or acquired (infectious) nature (Prusiner and DeArmond, 1994; Gambetti et al., 2003) (Table 1.1). Acquired TSEs are characterised by a prolonged, asymptomatic incubation period which is generally followed by a relatively short progressive clinical illness that invariably ends in death. Although a number of different types of TSE have been identified, the majority are associated with a common pathology generally confined to the CNS including a variable degree of spongiform vacuolation throughout the cerebral grey matter, reactive proliferation of astrocytes and microglial, neuronal loss and in certain TSEs the formation and deposition of amyloid plaques within the brain (Figure 1.1a-c) (Ironside, 1996; Budka, 2003). In addition, the advent of immunohistochemical techniques have shown that disease is normally, but not always, associated with the deposition of an abnormal and protease-resistant form of a normal host encoded-cell surface sialoglycoprotein, the prion protein (PrP), denoted PrP^{Sc} within CNS tissue and in some cases peripheral tissues of affected individuals (Figure 1.1d-f) (Bruce et al., 1989; van Keulen et al., 1996; Bell et al., 1997; van Keulen et al., 1999; Hill et al., 1999; Groschup et al., 1999; Head et al., 2004b). The relevance of the prion protein in these diseases is highlighted by the fact that TSEs have become most commonly referred to as “prion diseases”.

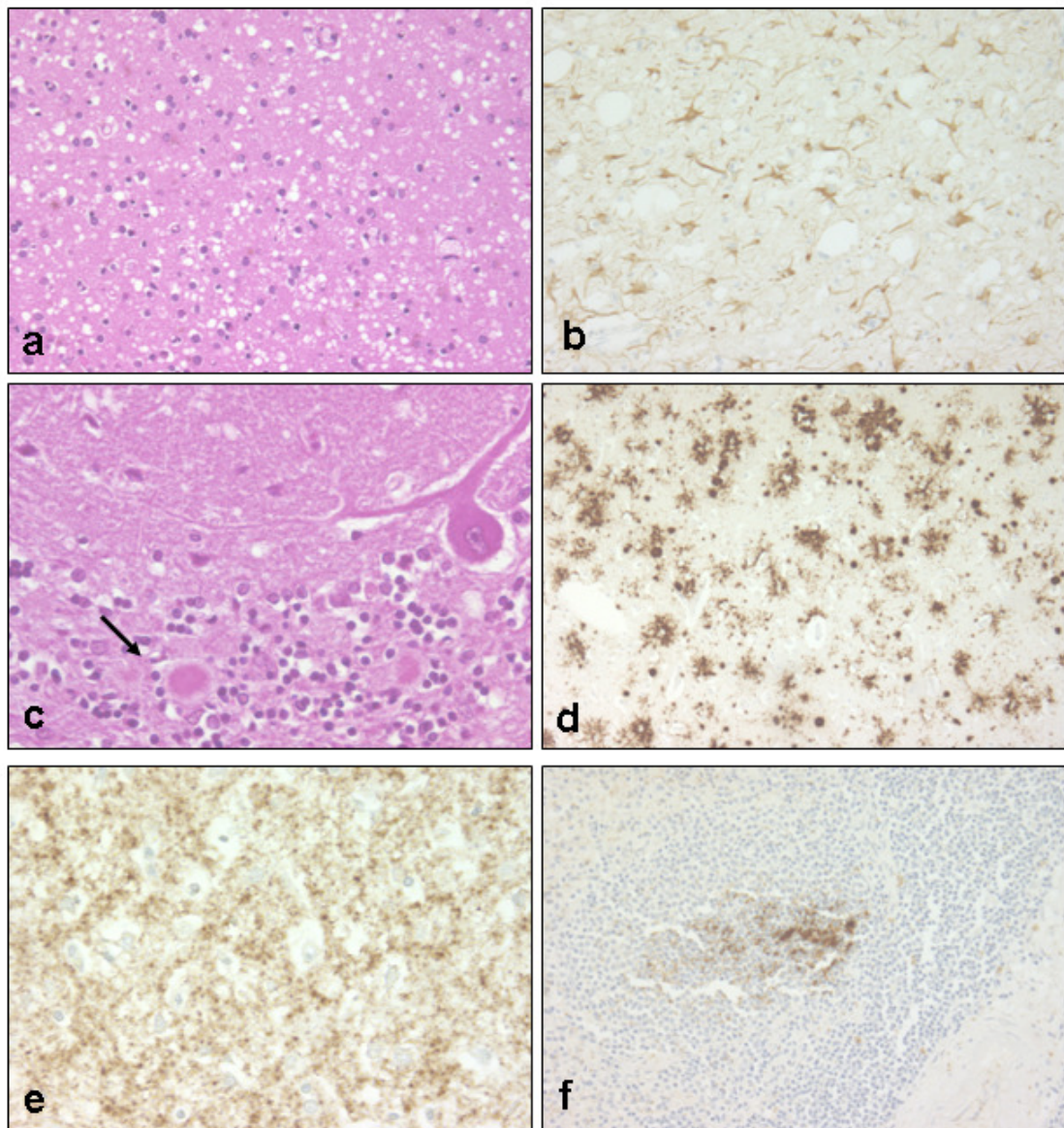
Table 1.1: TSEs of humans and animals

Aetiology	Disease	Natural host
Idiopathic	Sporadic Creutzfeldt-Jakob Disease (sCJD) ^a	Human
	Sporadic Fatal Insomnia (sFI) ^b	Human
	Variably protease-sensitive prionopathy(VPSPr) ^c	Human
Familial	Familial CJD (fCJD) ^d	Human
	Gerstmann-Sträusler-Scheinker (GSS) ^e	Human
	Fatal Familial Insomnia (FFI) ^f	Human
Acquired	Kuru ^g	Human
	Iatrogenic CJD (iCJD) ^h	Human
	Variant CJD (vCJD) ⁱ	Human
	Bovine Spongiform Encephalopathy (BSE) ^j	Cattle
	Feline Spongiform Encephalopathy (FSE) ^k	Cats (domestic and large)
	Exotic Ungulate Encephalopathy ^l	Nyala, Kudu etc
Probably acquired	Scrapie ^m	Sheep and goats
	Atypical scrapie ⁿ	Sheep
	Atypical BSE (H or L type) ^o	cattle
	Transmissible Mink Encephalopathy (TME) ^p	Mink
	Chronic Wasting Disease (CWD) ^q	Mule deer, white tailed deer and Elk

First reported; ^a(Creutzfeldt, 1920; Jakob, 1921), ^b(Mastrianni et al., 1997), ^c(Gambetti et al., 2008), ^d(Meggendorfer, 1930; Masters et al., 1981b), ^e(Gerstmann, 1928), ^f(Lugaresi et al., 1986), ^g(Zigas and Gajdusek, 1957), ^h(Duffy et al., 1974), ⁱ(Will et al., 1996), ^j(Wells et al., 1987), ^k(Wyatt et al., 1990), ^l(Kirkwood and Cunningham, 1994), ^m(Chelle, 1942), ⁿ(Benestad et al., 2003), ^o(Biacabe et al., 2004; Casalone et al., 2004), ^p(Hartsough and Burger, 1965; Casalone et al., 2004), ^q(Williams and Young, 1980).

Figure 1.1: Pathological features in TSEs

Pathological changes in human TSEs. **(a)** microvacuolar degeneration in the frontal cortex of an MM1 sporadic CJD case (haematoxylin and eosin stain). **(b)** Astrocytes immunolabelled for glial fibrillary acidic protein in the thalamus of a variant CJD case. **(c)** A kuru plaque (arrow) within the granular layer of the cerebellum in an MV2A sporadic CJD case (haematoxylin and eosin stain). Prion protein immunohistochemistry in frontal cortex tissue in **(d)** variant CJD and **(e)** iatrogenic CJD. **(f)** Prion protein immunohistochemistry within the spleen in variant CJD. Original magnification; x100 (d, f), x200 (a, b, e), x400 (c)



The first indication that TSEs may be transmissible came from the accidental transmission of scrapie in sheep following inoculation of sheep with a scrapie contaminated vaccine aimed at controlling louping ill (Gordon, 1946). The first evidence for the transmissibility of these diseases occurred during the 1930s with the successful transmission of scrapie in previously healthy sheep following intraocular injection with spinal cord tissue from sheep affected by scrapie (Cuillé and Chelle, 1936). Experimental transmission of scrapie to goats (Cuillé and Chelle, 1939) followed confirming the cross-species transmissibility of scrapie while demonstrating the unusually long incubation periods obtained in these experiments. However, it was not until 1959 and the work of William Hadlow, comparing the strikingly similar neuropathological features of scrapie with that of Kuru, an obscure neurological disease affecting the Fore-speaking people of the Eastern highlands of Papua New Guinea, that the first clues to the aetiology of human TSEs emerged (Hadlow, 1959). Subsequent transmission studies in which chimpanzees were inoculated with brain homogenate from kuru cases provided the first demonstration of the transmissible nature of human TSEs (Gajdusek et al., 1966). The inclusion of CJD as a TSE resulted from similar observations that the neuropathological features of kuru and CJD were remarkably similar (Klatzo et al., 1959). Again, it was experimental transmission studies which confirmed CJD as a member of the TSEs (Gibbs, Jr. et al., 1968).

A significant advance in TSE research came with the successful transmission of brain extracts from sheep scrapie to mice (Chandler, 1961) and subsequently to hamsters (Marsh and Kimberlin 1975, Kimberlin and Walker 1977). The development of rodent models provided, and continues to provide a practical model in which to study TSEs producing significantly shorter incubation periods (around 3-5 months) compared to that observed in sheep, goats and non-human primates (> 1 year) while maintaining high titres of infectivity in the brain. More recently, the generation of mice expressing novel transgenes has ushered a new era in the use of rodent models for the investigation of TSEs.

Mouse models play a key role in defining TSE strains. The existence of different strains of TSE was first suggested following the observation of two distinct clinical syndromes of scrapie in goats (“scratching” and “drowsy”) (Pattison and Millson, 1961), the clinical signs of which were reproduced on further transmission to goats. Following this, several distinct TSE strains have been identified in mice after serial passage of scrapie, BSE or CJD isolates from a range of sheep, goat, cattle or human sources. Such strains are distinguished by their unique biological properties, specifically incubation periods and patterns of neuropathological targeting (the “lesion profile”) after serial passage in mouse lines carrying different alleles of the mouse PrP gene (*Prn-p*) (Dickinson and Mackay, 1964; Dickinson et al., 1968; Bruce et al., 1991). These same strain-typing methods confirmed that the agent strain in vCJD was indistinguishable from that of BSE, providing compelling evidence that vCJD represented human infection with the BSE agent (Bruce et al., 1997).

Enormous public and scientific attention has focused on TSEs, not only because of their unique biological properties, but also more recently because of their impact on animal and public health, particularly with the appearance of BSE and vCJD in the United Kingdom. To date (December 2011), there have been a limited number of clinical cases (n=176) of vCJD in the UK (<http://www.cjd.ed.ac.uk/figures.htm>), much lower than first predicted and fears over an epidemic have receded. Concerns now focus on limiting the spread of vCJD by secondary human transmission from possible asymptomatic carriers, particularly through surgical procedures and surgical instruments, with the infectious agent showing an alarming resistance to conventional decontamination methods (Dunstan and Alpers, 2005). In addition, the observation that vCJD appears to be efficiently transmitted via transfusion with blood and blood products (Llewelyn et al., 2004; Peden et al., 2004; Wroe et al., 2006; Health Protection Agency., 2009; Peden et al., 2010) raises concerns over the secondary human transmission via this medical procedure. Such concerns have focused much of the current research on the development of a diagnostic and screening assay which could be applicable for use on biological fluids such as blood.

1.2 The infectious agent

Several hypotheses have been advanced to account for the atypical nature of the infectious agent causing TSEs. This remains an area of much controversy and one that has yet to be fully resolved. For many years the idea that these were “slow viruses” was accepted by much of the scientific community (Sigurdsson, 1954). Slow viruses were characterised by a prolonged incubation period of several months to decades; a brief, progressive clinical course leading to death; pathology confined to a single organ and a natural host usually confined to a single species, all of which are associated with scrapie (Sigurdsson, 1954). However, unlike most viruses, the scrapie agent showed resistance to many of the physical and chemical treatments that modify nucleic acids or decontaminate viruses. The resistance of the agent to formalin treatment (Gordon, 1946; Pattison, 1965), heat treatment (Alper et al., 1967) and the ability to withstand ultraviolet and ionizing radiation (Adams et al., 1969; Alper et al., 1978; Alper, 1985) suggested that the presence of a nucleic acid may not be essential, casting doubt that the agent is a virus. These observations led to a new focus and determination in elucidating the nature of the infectious agent and, a number of hypotheses were put forward, ranging from an abnormal, replicating polysaccharide with membranes (Gibbons and Hunter, 1967; Hunter et al., 1968) to a nucleoprotein complex (Latarjet et al., 1970), to a hybrid structure containing a host-independent informational molecule, protected by a host coded molecule (Bruce and Dickinson, 1987).

It was the British mathematician John Griffiths who first put forward the proposal that protein may be the causative agent of scrapie and other related TSEs. Griffiths suggested that under certain circumstances proteins might be able to self-replicate in the absence of nucleic acid. Two basic mechanisms were proposed, both involving the host's own protein. The first suggested that the scrapie agent was a protein with the ability to activate a damaging reaction normally suppressed in the host. The second proposed that the agent was an abnormal form of the host's own protein that could be spontaneously produced (Griffiths, 1967). Crucially, both these proposals allowed for the possibility that disease could occur spontaneously in previously healthy animals. Griffiths' work was the first account of what was to become known

as the 'protein-only' hypotheses. This proposal would remain unsupported until the 1980s, by which time it was clear that any hypothesis regarding the infectious agent would have to accommodate a number of distinct characteristics displayed by the agent. Not only would it have to explain the complex physico-chemical properties of the agent, but it would also have to account for the acquired, familial and sporadic forms of the disease. Furthermore, the existence of distinct TSE strains would also have to be accounted for by the 'protein-only' theory. It is this disease phenotype problem that has been, and continues to be, the most difficult to accommodate within a 'protein-only' hypothesis.

The 'protein-only' hypothesis was re-addressed and refined in the early 1980's by Stanley Prusiner with the development of a partial purification protocol for scrapie infectivity (Prusiner et al., 1981; Prusiner, 1982). By subjecting scrapie-infected hamster brain to treatments which modify nucleic acids or destroy viruses, Prusiner was able to further demonstrate that these treatments were unable to inactivate the scrapie agent. However, on exposing the same brain fractions to proteases, sodium dodecyl sulphate (SDS), phenol and urea; substances that are known to damage or destroy protein, the brain fractions became less or non-infectious (Prusiner et al., 1981; Prusiner, 1982). These findings lead Prusiner to deduce that the infectious agent must be composed of protein and a new term 'prion' was introduced to denote these unusual small *proteinaceous infectious* particles. Having shown that protein was a part of the infectious agent the search was on to isolate the protein or proteins that were present in the infectious agent.

1.2.1 The prion protein

Investigations of purified fractions from scrapie-infected hamster brain, identified a protease-resistant protein with a molecular mass of around 27-30 kiloDaltons (kDa), which was found in abundance in scrapie-infected brain tissue but which was absent in normal healthy control tissue (Bolton et al., 1982; Prusiner et al., 1982; McKinley et al., 1983). This unique protein was designated the prion protein (PrP) and termed PrP 27-30 after proteolytic treatment (Bolton et al., 1982). Further purification of the prion protein identified the N-terminal amino acid sequence (Prusiner et al., 1984)

from which oligonucleotides were synthesised to produce complimentary DNA (cDNA) (Oesch et al., 1985; Chesebro et al., 1985). Using this cDNA, a larger protein with a molecular mass of 33-35 kDa was identified. Investigation of messenger RNA levels of the prion protein identified showed similar levels in both the scrapie infected hamster brain and in the normal control tissue (Oesch et al., 1985). The discovery of a single gene, the human prion protein gene (*PRNP*) which encodes the prion protein, was a surprising and important discovery indicating that this was in fact a host encoded protein and not a viral protein as had been expected (Oesch et al., 1985; Chesebro et al., 1985). To distinguish between the two forms of the protein, the abnormal, disease-associated form was termed PrP^{Sc} for the scrapie-infected isoform or PrP^{res} for the protease resistant isoform after treatment with proteases (Caughey et al., 1990), whilst the normal cellular protease sensitive isoform was referred to as PrP^{C} or PrP^{sen} when defined by protease-sensitivity. More recently, the abnormal, and disease specific form of the protein has also been referred to as PrP^{d} to denote its disease specificity (Jeffrey et al., 2003) or PrP^{TSE} to denote TSE disease-specificity, irrespective of species and TSE agent involved (Herzog et al., 2005). However these later terms are less commonly used in the literature at present. For the purpose of this thesis the term PrP^{Sc} will be used when referring to the abnormal and disease-associated prion protein and PrP^{res} to designate PrP^{Sc} , when operationally defined by its resistance to proteolytic digestion, usually with, proteinase K.

The accumulation of PrP^{Sc} within CNS tissue is considered a hallmark of TSEs, whether in sporadic, familial or acquired forms. Indeed, the presence of PrP^{Sc} within CNS tissues is generally regarded as the surrogate marker for infectivity (Prusiner, 1998). Early investigations using infrared spectroscopy, circular dichroism and computer modelling demonstrated that, PrP^{C} and PrP^{Sc} have an identical primary structure, differing only in their structural conformation (Caughey et al., 1991). PrP^{C} is rich in α -helix content (40%) with little (3%) or no β -sheet content, whereas PrP^{Sc} is dominated by β -sheet (45%) with a much smaller α -helix content (20%) (Pan et al., 1993; Safar et al., 1993) (Figure 1.2). This basic structure of PrP is also found to be highly conserved between different species. The change in conformation alters the

physico-chemical properties of the prion protein in terms of degradation by proteinase K (and other proteases) and solubility in non-ionic detergents. In normal healthy cells, PrP^C is susceptible to digestion with proteases and is solubilized with non-ionic detergents, whereas in scrapie infected tissue, PrP^{Sc} demonstrates a partial resistance to protease treatment and polymerises into amyloid rods after detergent extraction (Oesch et al., 1985; Meyer et al., 1986). The changes, both structural and biochemical, which occur during the conversion of PrP^C to PrP^{Sc} are summarised in Table 1.2.

Figure 1.2: Model of primary structure of PrP^C and PrP^{Sc}

PrP^C is composed of mainly of α -helical structures (red shaped spirals) whereas PrP^{Sc} is remodelled into a β -helical structure (green rods). N-terminus and C-terminus are not shown (adapted from Soto et al 2001).

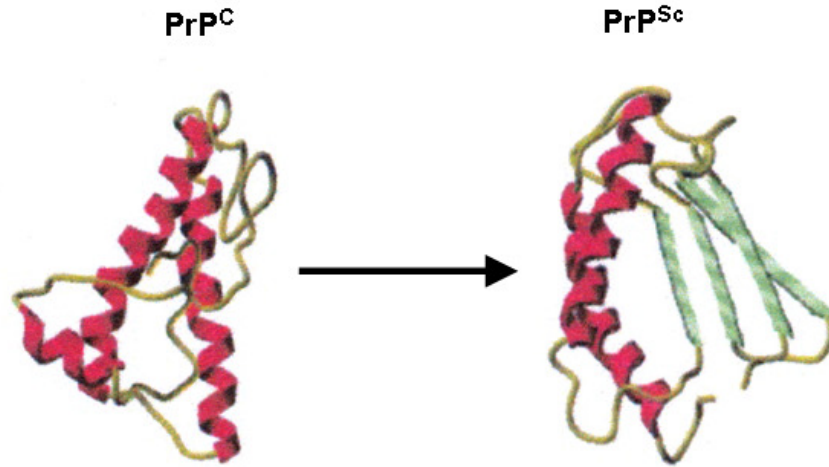


Table 1.2: Biochemical and physical properties of PrP^C and PrP^{Sc}

PrP ^C	PrP ^{Sc}
Present in normal tissue	Present in TSE affected tissue only
M _r 33-35 kDa	M _r 33-35 kDa
Predominantly α -helix structure	Increased β -sheet structure
Sensitive to protease treatment	Protease resistant core M _r ~27-30
Soluble in detergents	Insoluble in detergents forming aggregates
Attached to cell surface by a GPI anchor	Accumulates on the cell surface or internalised within cells or as extracellular fibrils, plaques or amorphous deposits
GPI anchor sensitive to cleavage with phosphatidylinositol-specific phospholipase C (PIPLC)	GPI anchor only sensitive to cleavage after denaturation
Rapid synthesis and turn over	Accumulation slow, resistant to degradation

1.3 The prion protein gene

A single gene, the prion protein gene was found to encode PrP^C (Oesch et al., 1985). This gene was found to be highly conserved within mammals, with only minor differences in the amino acid sequence between species (Prusiner et al., 1998). This gene plays a key role in TSEs, with polymorphisms in the coding region in mouse and human that influences the incubation period and disease susceptibility. The prion protein gene in human and in mouse is described in detail in the following section. Three further genes have been identified; Doppel (*PRND*) (Moore et al., 1999), Shadoo (*SPRN*) (Premzl et al., 2003) and *PRNT* (Makrinou et al., 2002), which are related to the prion gene in terms of sequence homology, and in two of the genes by genomic location. However, direct involvement of these three genes in TSEs remains to be shown.

1.3.1 The human prion protein gene

The human prion protein gene, *PRNP*, was first described in 1986 (Liao et al., 1986) and is a single copy gene located on the short arm of chromosome 20. The gene contains two exons separated by a 10kb intron with the entire protein-coding region of the prion protein contained within the second exon, encoding a protein of 253 amino acids (Puckett et al., 1991). More than 20 distinct point mutations together with insertions and deletions within the octapeptide repeat region have been identified within the human PrP gene which are directly linked with the familial forms of human prion disease (Prusiner and Scott, 1997; Kovacs et al., 2002b; Mead, 2006) (Figure 1.3). The role these mutations in the *PRNP* gene play in the development of disease is unclear. However, it is possible that these mutations either cause the prion protein to become less stable making it more likely to cause the conversion of PrP^C to PrP^{Sc} (Prusiner, 1997) or that individuals with such mutations become more susceptible to TSE infection (Manson et al., 1999; Chesebro, 1999).

1.3.1.1 PRNP codon 129 polymorphism

In addition to mutations in the *PRNP* gene, numerous polymorphisms have been identified in the PrP gene. Polymorphisms differ to mutations in TSEs in that they do not directly cause disease but rather affect the susceptibility of an individual to disease or alter the course of the disease. In humans, a naturally occurring polymorphism found at codon 129 on *PRNP* is a recognised risk factor for human TSEs either on its own or in conjunction with other mutations in the prion protein. It has important effects on the susceptibility to disease, clinical manifestations and the incubation period (in acquired forms) (Palmer et al., 1991; Collinge et al., 1991; Parchi et al., 1996; Zeidler et al., 1997; Deslys et al., 1998; Alperovitch et al., 1999; Lee et al., 2001; Brandel et al., 2003). At codon 129, an ATG or GTC amino acid sequence results in either the presence of methionine (M) or valine (V). The presence of M or V at codon 129 can be either homozygous or heterozygous. Both M and V homozygotes are over represented in human TSEs when compared to the normal population while heterozygotes are under represented (Palmer et al., 1991; Alperovitch et al., 1999). In sporadic CJD (sCJD), analysis of *PRNP* has demonstrated that around 70% of all cases reported are found in M homozygous individuals (Alperovitch et al., 1999) with this figure rising to 100% in the case of vCJD (Bishop et al., 2009). These figures have been shown to be statistically significant when compared to the 40% normal population frequencies for the MM genotype (Collinge et al., 1991; Nurmi et al., 2003). Therefore, homozygosity for methionine is clearly a risk factor in the development of sCJD and vCJD. PrP codon 129 distributions in sporadic and vCJD compared with the normal UK population are shown in Table 1.3.

The *PRNP* codon 129 polymorphism is also believed to play an important role in disease phenotype as demonstrated in sCJD (Parchi et al., 1996) and in cases of familial disease, most clearly demonstrated in the D178N mutation. In these familial cases, a mis-sense mutation at codon 178 coupled with the presence of M at codon 129 on the mutant allele results in the clinical picture of fatal familial insomnia (FFI). Whereas, the same mutation at codon 178 when coupled with V at codon 129 on the mutant allele on *PRNP* results in the disease phenotype of familial CJD (fCJD)

(Monari et al., 1994; Cortelli et al., 1999). The role that *PRNP* codon 129 plays in the disease phenotype in sCJD is discussed later on in Chapter 1.

Figure 1.3: Mutations and polymorphic variants of the human *PRNP*

The human prion protein gene showing positions of common pathogenic mutations (red) and non-pathogenic polymorphisms (green). (By kind permission of Matthew Bishop)

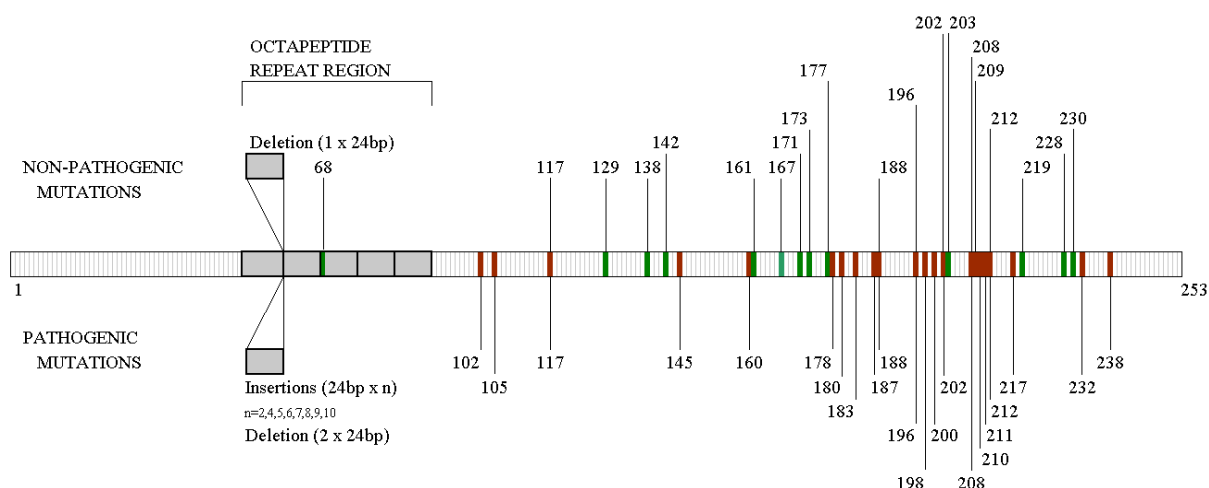


Table 1.3: PrP codon 129 genotype distributions

	MM (%)	MV (%)	VV (%)
*Normal population	42	47	11
†sCJD (UK 1990-2002)	68	16	16
†vCJD	100	0	0

* Codon 129 genotype frequency data from Nurmi *et al.* (Nurmi et al., 2003)

† Codon 129 genotype frequency data from Knight and Will (Knight and Will, 2004)

1.3.2 The mouse prion protein gene

The mouse prion protein gene maps to the homologous region of chromosome 20 (Sparkes et al., 1986; Lee et al., 1998) and is designated *Prn-p*. The mouse PrP gene has a third exon which contains the entire open reading frame encoding a protein of 254 amino acids long. In the mouse, three alleles of the *Prn-p* gene have been identified, encoding proteins that differ by two amino acids at codon 108 and 189; these are *Prn-p^a* (Leu-108, Thr-189), *Prn-p^b* (Phe-108, Val-189) and *Prn-p^c* (Phe-108, Thr-189) (Westaway et al., 1987; Lloyd et al., 2004b). The vast majority of mouse lines analysed, and those used in this thesis, are either from the *Prn-p^a* or *Prn-p^b* genotype (Westaway et al., 1987).

It is now confirmed that the mouse *Prn-p* gene is congruent to another gene identified in the mouse, the *Sinc* gene, which had been identified much earlier (Dickinson et al., 1968). The *Sinc* gene (short for scrapie incubation) was found to have a major influence on the incubation period of scrapie and other TSEs in mice. The effect of the *Sinc* gene was first demonstrated with the experimental transmission of the ME7 scrapie strain in which two alleles of the *Sinc* gene, designated s7 and p7, produced short and long incubation periods, respectively (Dickinson and Mackay, 1964; Dickinson et al., 1968). Subsequent studies showed that the incubation period in other TSE strains were also controlled by the *Sinc* gene (Dickinson and Meikle, 1971; Bruce et al., 1991). It is now established that the *Sinc^{s7}* allele corresponds to *Prn-p^a* with the *Sinc^{p7}* allele corresponding to *Prn-p^b* (Hunter et al., 1992; Moore et al., 1998). In the routine strain typing of different TSE isolates, groups of *Sinc^{s7}/Prn-p^a*, *Sinc^{p7}/Prn-p^b*, and *Sinc^{s7p7}/Prn-p^{ab}* mice are included which results in highly reproducible patterns of incubation period in each mouse *Prn-p* genotype (Bruce et al., 1991).

1.4 Cell biology and function of PrP^C

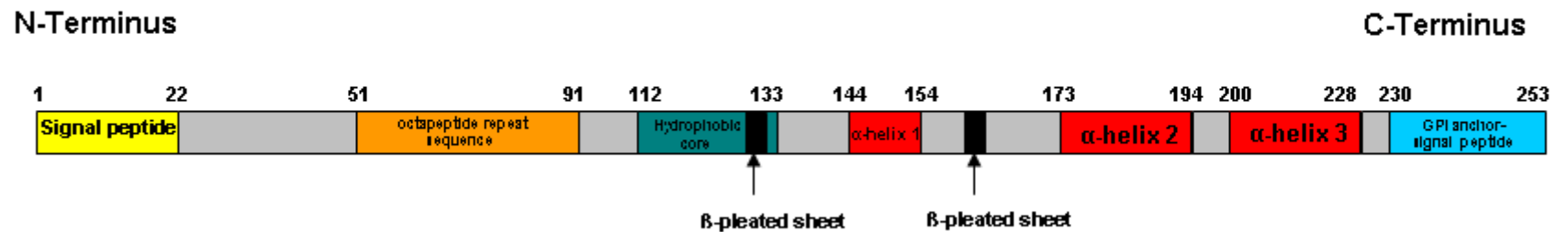
PrP^C is a glycosyl-phosphatidylinositol (GPI)-linked cell surface membrane protein which is expressed early in embryogenesis and which reaches adult levels soon after birth (Kretzschmar et al., 1986; Manson et al., 1992). PrP^C is predominantly expressed in neuronal cells of the brain and spinal cord, but has been found at lower levels in a wide range of peripheral tissues (Linden et al., 2008). The protein, which is encoded by the prion protein gene, contains several distinct domains including an N-terminus, which has a series of five proline and lysine-rich octapeptide repeats, a highly conserved central hydrophobic region and a C-terminal globular domain (Figure 1.4). NMR studies have shown that the N-terminus of the prion protein is very flexible and lacks any secondary structure, whereas the C-terminus region contains a well-defined folded domain (Prusiner, 1998). The central region of the protein is arranged in three α -helices (residues 144-154, residues 173-194 and residues 200-228) interspersed with two β -pleated sheet formations (residues 128-131 and residues 161-164). A single di-sulphide bond is present between Cysteine residues 179 and 214 in α -helix two and three respectively. In addition, PrP^C contains two consensus sequences for N-linked glycosylation (Asn 181 and Asn 197) and can be found in the cell in the un-, mono- and di-glycosylated forms (Stahl and Prusiner, 1991).

PrP^C is highly conserved between species suggesting that the prion protein plays a fundamental physiological role. Defining this role may be crucial in the understanding of TSEs, since the protein may fail to perform its normal function once converted to the disease-associated form. Much work has been carried out on the phenotype of PrP null mice, in which the *Prn-p* gene has been inactivated or deleted. PrP null mice are resistant to scrapie challenge and show no signs of infectivity or accumulation of PrP^{Sc} after inoculation with TSEs (Bueler et al., 1993; Sailer et al., 1994). The generation of different PrP null mice has resulted in the demonstration of different phenotypes. Some studies reported no obvious abnormalities in the phenotype of null mice, other than a resistance to prion infection (Bueler et al., 1993; Manson et al., 1994), whereas others reported several

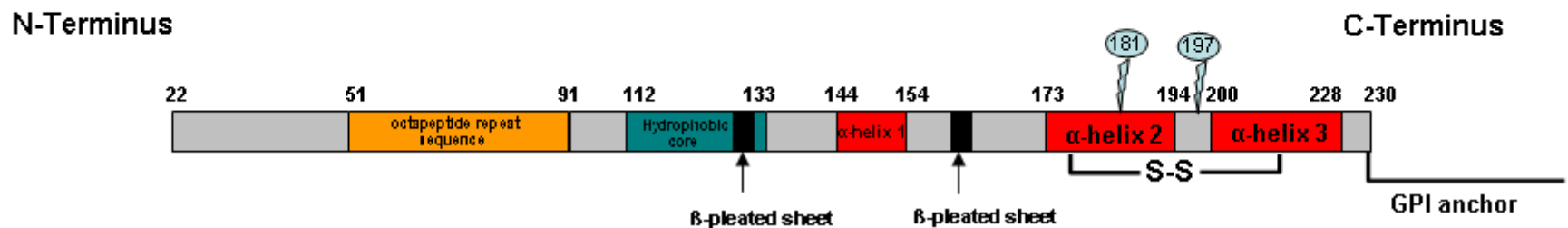
Figure 1.4: Structure of the human prion protein

Schematic diagram of the structure of the cellular prion protein following (a) synthesis and (b) after post-translational modification. The numbers correspond to the positions of the amino acids in the human prion protein. The two N-glycan attachment at amino acids 181 and 197 are shown in blue circles. The β -pleated sheet regions are present at residues 128-131 and residues 161-164.

(a)



(b)



neurological abnormalities including; disturbance of synaptic transmission (Collinge et al., 1994), nerve cell organisation (Colling et al., 1997) and spatial learning (Criado et al., 2005), and alterations in circadian rhythm, sleep pattern (Tobler et al., 1996) and olfaction (Le Pichon et al., 2009).

Although the normal function of PrP^C is not known, a number of roles for PrP^C have been proposed. Like many membrane bound proteins, PrP^C is synthesised and co-translocated into the rough endoplasmic reticulum (ER), transits the Golgi apparatus, before appearing attached to the cell surface where it resides predominantly in lipid rafts (Harris, 2003). The cellular localisation of PrP^C would be consistent with a role for PrP^C as a membrane receptor, cell adhesion molecule (Schmitt-Ulms et al., 2001; Mange et al., 2002) or linked to a signal transduction pathway (Spielhaupter 2001, Mouillet-Richard 2000). As PrP^C expression is not restricted to neurons it is unlikely that it has a purely synaptic function. There is increasing evidence that PrP^C may have a role in copper metabolism. The binding of metals, in particular copper ions (Cu²⁺), indicates that PrP^C may be a metal-binding protein functioning as a metal transporter (Brown et al., 1997a; Stockel et al., 1998; Kramer et al., 2001). Several lines of experimental evidence indicate that PrP^C has a role in protecting cells against oxidative stress. Cerebellar granular or neocortical neurons cultured from PrP null mice were found to be more susceptible to treatments that induce oxidative stress, including copper ions and hydrogen peroxide when compared to neurons cultured from wild-type mice (Brown et al., 1997b; Brown et al., 2002). Consistent with these studies, scrapie-infected brain tissue has been shown to have increased levels of oxidative stress markers (Wong et al., 2001). Later studies confirmed that the brain infarct induced by hypoxia or ischemia are significantly larger in PrP null compared to wild-type mice (McLennan et al., 2004; Sakurai-Yamashita et al., 2005; Spudich et al., 2005). More recently, two studies have proposed a role for PrP^C in the proliferation and differentiation of hematopoietic stem cells (Zhang et al., 2006) and neural precursor cells (Steele et al., 2006). The studies described all suggest that TSEs could result from the loss of function of PrP^C. However, as the phenotype of null mice is so mild, disease resulting from a gain in function from the accumulation PrP^{Sc} is also possible

1.5 The prion hypothesis

The pathogenesis of TSEs is associated with the conversion of PrP^C to PrP^{Sc}. The mechanism or mechanisms underlying this protein conversion are yet to be fully resolved, but have been attributed to conformational changes in which portions of the α -helical and coil structure of PrP^C are refolded into a β -sheet complex. At present, the prion hypothesis, which is refined from the 'protein only' theory, is the most widely accepted of the models put forward but remains to be conclusively proven (Prusiner, 1991). According to the prion hypothesis, PrP^{Sc} is the principal and possibly the sole constituent of the transmissible agent. It predicts that replication occurs through the post-translational conversion of PrP^C to PrP^{Sc} via direct interaction of the two proteins resulting in the generation of further PrP^{Sc}. PrP^{Sc} therefore acts as a conformational template, promoting the conversion of PrP^C to the abnormal and disease-associated form of the protein. This conversion initiates a chain reaction in which the newly formed PrP^{Sc} converts further PrP^C into the disease-associated form. This hypothesis proposes that it is the accumulation of PrP^{Sc} within the brain that results in the characteristic pathology of TSEs, which in turn leads to the presentation of clinical symptoms.

An important and early support for a link between PrP and the development of TSE disease came from the identification and cloning of the *PRNP* gene (Oesch et al., 1985) and the observation that inherited forms of human TSEs were consistently associated with point mutations in the *PRNP* gene (Hsiao et al., 1989; Doh-ura et al., 1989; Goldgaber et al., 1989; Goldfarb et al., 1990; Hsiao et al., 1991; Goldfarb et al., 1991). However, the most convincing support for the prion hypothesis came from the finding that PrP null mice were completely resistant to scrapie infection (Bueler et al., 1993; Sailer et al., 1994). Furthermore, susceptibility to scrapie infection in these PrP null mice is fully restored with the introduction of the murine PrP gene (*Prn-p*) (Brandner et al., 1996; Fischer et al., 1996). The resistance of PrP null mice to scrapie infection demonstrates that PrP is a vital requirement in the pathogenesis of the disease but does not rule out the possibility that PrP may simply be a receptor for the agent.

The development of *in vitro* models for the generation of infectious PrP aggregates has provided the most recent support for the prion hypothesis. The earliest *in vitro* models demonstrated that under certain conditions, PrP^C generated from cultured cells could be transformed into PrP^{Sc} when mixed with PrP^{Sc} from scrapie-infected brain samples (Kocisko et al., 1994; Caughey et al., 1995). These experiments demonstrated that there are two steps in the conversion of PrP^C to PrP^{Sc}; firstly, the binding of PrP^C to the PrP^{Sc} oligomer and secondly the conversion of the bound PrP^C to the disease-associated conformation. However, these early reactions suffered from low yields of newly generated PrP^{Sc} making it difficult to assess any infectivity present in the *in vitro* generated protein. The demonstration of infectivity and transmissibility from PrP^{Sc} generated *in vitro* would provide further convincing support for the acceptance of the prion hypothesis.

Recently, two novel methods of generating PrP^{Sc} *in vitro* have demonstrated TSE infectivity associated with the generated PrP^{Sc}. In the first study, TSE infectivity was demonstrated in transgenic mice over-expressing a truncated form of PrP (Tg9949) following inoculation with PrP aggregates ('synthetic prions') generated by refolding of recombinant PrP (Legname et al., 2004; Legname et al., 2005). However, given that these synthetic prions were only infectious to this transgenic model and the resulting infectious titres obtained were very low, the possibility that the infectivity was a result of spontaneous disease in these PrP over expressing mice can not be discounted. Further support for the prion hypothesis has come from studies using the protein misfolding cyclic amplification (PMCA) system for the *in vitro* amplification of PrP^{Sc}. In PMCA, a sample containing PrP^{Sc} (seed) is diluted into a substrate containing excess PrP^C (substrate). Using successive rounds of sonication and incubation, the PrP^{Sc} seed is amplified at an accelerated rate (Saborio et al., 2001). PMCA has been successfully applied to a number of animal models of TSE using substrates prepared from either PrP^C from brain tissue (Castilla et al., 2005; Weber et al., 2006; Castilla et al., 2008; Green et al., 2008); PrP^C purified from brain (Deleault et al., 2003; Deleault et al., 2005; Deleault et al., 2007) or bacterially expressed purified recombinant PrP (Kim et al., 2010; Wang et al., 2010). Infectivity has been successfully demonstrated with the PMCA amplified products. However,

all these studies suggest that the successful conversion of PrP^C to PrP^{Sc} may require either an additional seed of PrP^{Sc} (Kim et al., 2010) or other cofactors such as phospholipids or polyanionic molecules (such as RNA) (Deleault et al., 2003; Deleault et al., 2005; Deleault et al., 2007; Wang et al., 2010). Nevertheless, the generation of PrP^{Sc} *in vitro* has gone a long way in providing support for the prion hypothesis demonstrating that the crucial step in TSEs is the conformational transmission of PrP^{Sc}. The precise molecular mechanism of conversion of PrP^C to PrP^{Sc} remains unclear; however, two models have been proposed (Figure 1.5).

1.5.1 The ‘template assisted conversion’ model

The first was originally described as the ‘template assisted conversion model’ (Prusiner, 1991; Telling et al., 1995). This model (Figure 1.5a) postulates that PrP^{Sc} is inherently more stable than the normal cellular protein PrP^C. However, there is a kinetic barrier before reaching this stable conformation. This model, proposes that a transient conformational intermediate exists (PrP*) in equilibrium with PrP^C. This intermediate form has the ability to heterodimerize with PrP^{Sc} monomers after binding to a molecular chaperone, postulated as another protein termed protein X (Telling et al., 1995; Prusiner, 1997; Kaneko et al., 1997). This heterodimer is able to spontaneously convert into a PrP^{Sc} homodimer consisting of the old and newly formed PrP^{Sc}. Once produced PrP^{Sc} is then able to induce further conversion of PrP^C to PrP^{Sc} generating an exponential growth of PrP^{Sc}. Genetic mutations in *PRNP*, may influence a shift in the levels of PrP^C and PrP*, favouring PrP*. This would present an increased risk in the formation of PrP^{Sc}. However, to date the majority of experimental data suggests that PrP conversion is associated with the aggregation process, with little or no evidence of a stable PrP^{Sc} monomer.

1.5.2 The ‘nucleation-polymerization’ model

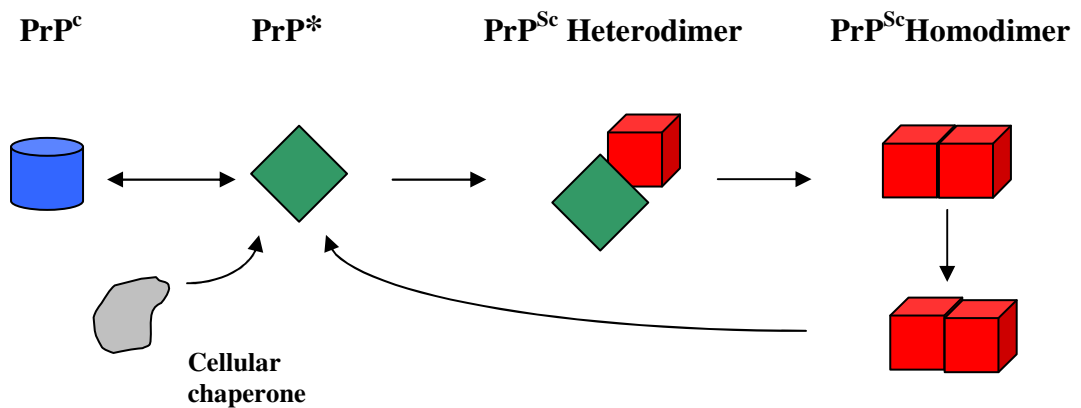
The second model is described in the “nucleation-polymerization model” (Jarrett and Lansbury, Jr., 1993). This model (Figure 1.5b) proposes that PrP^C and PrP^{Sc} coexist in relative equilibrium. In this model, PrP^{Sc} has a relatively unstable conformation, which becomes stabilized upon aggregation with other PrP^{Sc} molecules. The presence of PrP^{Sc} aggregates acts as a seed, promoting the conversion of further

PrP^{Sc} by binding to and stabilizing the otherwise favoured PrP^C. This displaces the equilibrium between PrP^C and PrP^{Sc} favouring the formation of the pathological conformer. The rate limiting step in this model is the initial nucleation process in which the production of PrP^{Sc} and in turn, PrP^{Sc} aggregates is not favoured. Support for this model is described earlier in this section with the demonstration that PrP^C can be converted to PrP^{Sc} by incubation with PrP^{Sc} from infected animals (Kocisko et al., 1994; Caughey et al., 1995) and more recently from amplification of PrP^{Sc} *in vitro* using PMCA (Castilla et al., 2005; Weber et al., 2006; Castilla et al., 2008; Green et al., 2008). In inherited TSEs, it is believed that point mutations in the prion protein gene, favour the spontaneous conversion of PrP^C into PrP^{Sc} without the need for contact with the infectious agent. Sporadic forms of the disease, which include around 85% of all CJD cases, may be a result of either spontaneous conversion of PrP^C or as a result of an undetected somatic mutation in the protein favouring the conversion of PrP^C to PrP^{Sc}.

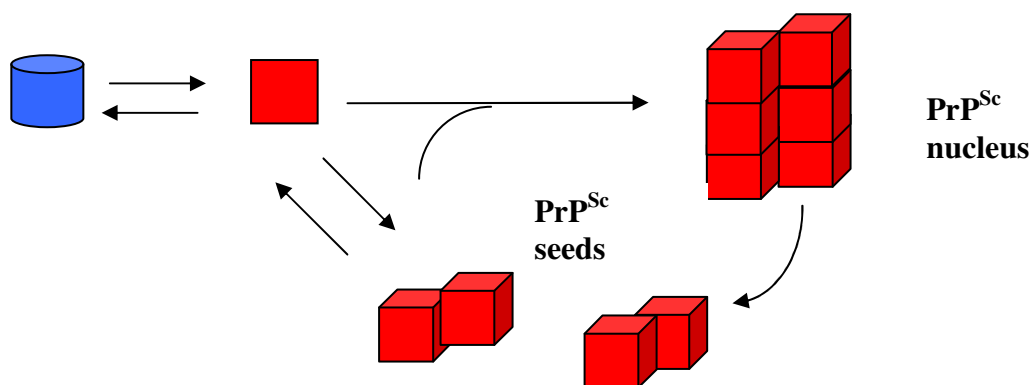
Figure 1.5: Schematic diagrams for different models of prion replication

Theoretical models of PrP^{Sc} formation. **(a)** In the 'template assisted conversion model' the key step is in the formation of PrP^{Sc} , which acts as a template for further conversion. **(b)** In the 'nucleation polymerisation model' the key step is in the formation of PrP^{Sc} aggregates which act as a seed for the further formation of PrP^{Sc} aggregates. This moves the equilibrium towards formation of the pathological protein. See text for details.

(a) The template assisted conversion model



(b) Nucleation polymerisation model



1.6 The virino hypothesis

An alternative to the prion hypothesis which is supported by some TSE researchers is outlined in the ‘virino’ theory (Bruce and Dickinson, 1987; Dickinson and Outram, 1988; Hope, 1994; Farquhar et al., 1998). This theory predicted the involvement of a host encoded component in the infectious agent, which is recruited into the infectious particle by a host-independent informational molecule yet to be identified. This hypothetical molecule, most probably a small, non-translated nucleic acid has the ability of carrying strain-specific genetic information whilst protected against degradation and inactivation by a host protein, PrP. Although this would fulfil all the biological requirements for the agent, attempts to identify TSE-specific nucleic acids have as yet proved unsuccessful.

1.7 Strain diversity

The demonstration of distinct TSE strains, a feature shared with nucleic acid-based pathogens, remains a challenge to the overall acceptance to the prion hypothesis. The existence of unique strains questions how a single misfolded protein that lacks any detectable nucleic acid can encipher all the information for multiple phenotypes within a single species. Such strain diversity found within TSEs continues to be the main supporting evidence for an independently replicating informational molecule or genome (Bruce and Dickinson, 1987).

Research by Alan Dickinson and co-workers demonstrated that distinct disease phenotypes could be raised and propagated in lines of inbred mice following experimental challenge with scrapie isolates (Dickinson et al., 1968; Fraser and Dickinson, 1973). Historically, these individual rodent-adapted TSE strains or varieties of TSEs are differentiated by a number of well defined clinical and pathological ‘strain typing’ methods that depend primarily on incubation period of the disease following inoculation (Dickinson et al., 1968) and regional patterns of spongiform change in nine defined brain regions which is referred to as the ‘lesion profile’ (Fraser and Dickinson, 1973). More recently, variations in the pattern of PrP^{Sc} deposition in mice following challenge with TSE isolates has also been associated with variations in strain (Hecker et al., 1992; DeArmond et al., 1993).

1.7.1 Incubation period

The incubation period in mice experimentally challenged with different TSE agents is defined as the period between initial infection and the clinical end-point of disease which is determined by a range of clinical presentations (Dickinson et al., 1968). In non-transgenic (wild-type) mice incubation period can range between 4 months and the life span of the mouse (over 2 years). In addition to strain, incubation period in mice is influenced by route of inoculation and dose. The *Prn-p* genotype of the mouse is also a major determinant of incubation period as described previously (Chapter 1, section 1.3.2) and can make a difference of hundreds of days to the incubation period of a single TSE strain. In some strains the shortest incubation period occurs in *Prn-p^a* mice whereas with other strains *Prn-p^b* mice produce the shortest incubation period. It is often the case that the incubation period in *Prn-p^{ab}* mice lies somewhere between the two homozygotes. Transmission of individual TSE agents into mice with the three different PrP genotypes, produce highly reproducible incubation periods, often sufficient on their own to identify the strain of agent.

1.7.2 Lesion profile

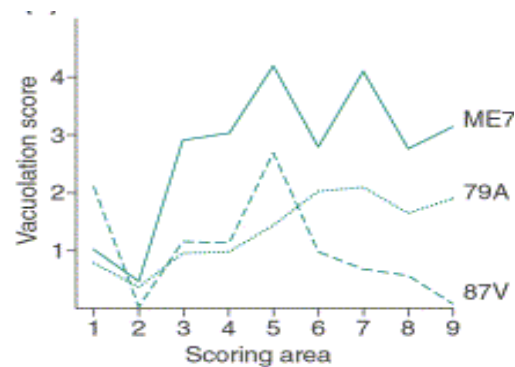
The lesion profile depends on the assessment of severity and distribution of TSE-associated spongiform change or vacuolation within the brain of the challenged mice (Fraser and Dickinson, 1968). For construction of a lesion profile, a semi-quantitative assessment is made of the severity of vacuolation in nine anatomically defined regions of the grey matter and three defined white matter regions of the infected mouse, known as the scoring regions (detailed in Chapter 2, section 2.6.3). Mean scores for each area are then plotted to produce a characteristic 'lesion profile' (Fraser and Dickinson, 1968). An example of the lesion profiles for three scrapie strains (ME7, 87V and 79A) in the grey matter regions in a single *Prn-p^b* (VM) mouse line is shown in Figure 1.6.

Together the incubation period and lesion profile in an inbred mouse line provides a 'signature' of the biological properties of the TSE agent. A number of distinct strains derived from natural sheep scrapie have been identified through incubation period and lesion profiling whereas passage of cattle BSE through panels of inbred mice has

indicated that BSE is associated with a single strain of agent (Bruce et al., 1994). Lesion profiling along with incubation period played a pivotal role in establishing the relationship between vCJD in humans and BSE. Transmission of vCJD to a panel of inbred mice produced a lesion profile which differed greatly from those of scrapie and sCJD, but which was indistinguishable from that of BSE (Bruce et al., 1997).

Figure 1.6: Examples of lesion profiles in mouse adapted scrapie strains

Lesion profiles for the ME7, 79A and 87V mouse adapted scrapie strains in a single *Prn-p^b* mouse line (VM). Adapted from Baron 2002.



1.7.3 Molecular basis of strains

As TSE strains can be propagated in mice with the same *Prn-p* genotype, it is clear that strain-specific information can not be encoded by differences in the PrP amino acid sequence. Furthermore, a defined strain can be re-isolated in mice after passage in intermediate species with different PrP sequences (Bruce et al., 1994). In order to be accommodated within the prion hypothesis, it has been proposed that strain-specific properties are enciphered by differences in the biochemical properties in the propagated PrP^{Sc}, specifically in the different conformation and glycosylation states of PrP^{Sc} (Collinge et al., 1996; Telling et al., 1996).

In seminal experiments, Bessen and Marsh identified two strains of transmissible mink encephalopathy (TME); the so-called ‘hyper’ (HY) and ‘drowsy’ (DY) strains. These two strains were identified by differences in their behavioural phenotype, incubation period and pathological profile after passage of a single TME isolate in Syrian hamsters (Bessen and Marsh, 1992b). In addition, strain-specific differences were observed in the PK resistant cores of the PrP^{Sc} associated with these different TME strains. These differences were represented by different migration patterns of PrP^{res} on polyacrylamide gels, and were attributed to differences in the N-terminal truncation sites of HY and DY PrP^{Sc}, clearly implying that HY and DY have different PrP^{Sc} conformations (Bessen and Marsh, 1992a; Bessen and Marsh, 1994). Such strain-specific differences in the physico-chemical properties of PrP^{Sc} were further demonstrated with the experimental transmission of two forms of familial CJD into a transgenic mouse model (Telling et al., 1996). In this study, inoculation of brain homogenate from a fatal familial insomnia (FFI) patient into transgenic mice expressing a chimeric human-mouse PrP gene (TgMHu2M) resulted in an incubation period of ~200 days with a ~19kDa PrP^{res} fragment after Western blot analysis. In the same study, inoculation of brain homogenate from a familial CJD (fCJD) patient into the TgMHu2M mice also resulted in an incubation period of ~200 days; however, Western blot analysis resulted in a ~21kDa PrP^{res} fragment. These findings demonstrated that the TgMHu2M PrP^{Sc} can exist in two different conformational states as determined by the size of the protease-resistant fragment (PrP^{res}). Furthermore, the different PrP^{res} fragments are associated with different human TSE

strains. Further evidence for strain-specific differences in PrP^{Sc} conformation have been provided by findings of studies showing differences among PrP^{Sc} aggregates in relation to the exposure of certain epitopes during guanidine induced denaturation (Safar et al., 1998), positions and intensities of infrared bands associated with β -sheet structure (Caughey et al., 1998) and sensitivity to protease digestion denaturation by guanidine HCL (Peretz et al., 2002).

Strain-specific differences have also been observed in the glycosylation of asparagine residues at positions 181 and 197 (on the human PrP sequences). Distinct TSE strains have consistent ratios of the di-, mono-, and unglycosylated forms of PrP^{res}. These distinct glycosylation profiles are conserved on serial passage in animals, suggesting that different PrP^{Sc} aggregates can selectively recruit PrP^C monomers in particular ratios according to glycosylation state (Prusiner, 1998; Collinge, 2001).

Several distinct human PrP^{res} types have been identified based on differences in the electrophoretic mobility (fragment size) of PrP^{res} and glycosylation ratio after PK digestion and these have been associated with different disease phenotypes of CJD (Collinge et al., 1996; Parchi et al., 1996). These different PrP^{res} types are proposed to be the molecular basis of strain typing. However, the relationship between PrP^{res} types and agent strain remains unclear and has been recently questioned by the observation of mixed PrP^{res} types within the brain of a proportion of sCJD cases (Parchi et al., 1999; Puoti et al., 1999; Schoch et al., 2006; Uro-Coste et al., 2008). Nevertheless, molecular typing continues to play an important role in the diagnosis of human TSE. For these different PrP^{res} types to be considered as the biochemical basis of agent strain; the biological and biochemical characteristics of the agent must be retained after transmission to both the same species and that of other species. The relationship between PrP^{res} type and disease phenotype in CJD will be fully discussed later in this Chapter (Chapter 1, section 1.10).

1.8 The species barrier

Another important issue related to prion strains is the mechanism of the ‘species barrier’ (Pattison, 1965; Pattison and Jones, 1968). The ‘species barrier’ in TSEs is defined as the relative resistance to TSE disease by one species following infection with TSE agent of another species. In some instances the ‘species barrier’ provides complete resistance to disease. This is best illustrated with hamster scrapie strain 263K, which does not transmit disease to mice despite the short incubation period of 65 days when passaged in hamsters (Kimberlin and Marsh, 1975). More often the ‘species barrier’ is reflected by longer more irregular incubation periods (Chandler, 1961; Dickinson et al., 1975). For example, the Chandler strain of mouse scrapie can be transmitted to mice with an incubation period of around 120 days, while Syrian hamsters have an incubation period of up to 380 days with the same inoculum suggesting a substantial ‘species barrier’ between the two species (Kimberlin and Walker, 1978; Kimberlin et al., 1987). Changes in the disease characteristics of the recipient and a reduction in the number of animals that succumb to disease are also indicative of the ‘species barrier’. However, as the agent is serially passaged through the new host, adaptation occurs which usually sees a drop in incubation period and a more constant pattern of clinical and neuropathological signs, which stabilise after further passages (Dickinson, 1976; Bruce et al., 1994).

The ‘species barrier’ is thought to be heavily influenced by the degree of homology in the primary PrP amino acid sequence between the PrP^{Sc} of the donor and PrP^C of the recipient species. This is best illustrated through studies using transgenic mouse models. In one such study, wild-type mice challenged intracerebrally with scrapie infected hamster tissue failed to show any signs of disease after around 500 days post injection, indicative of a ‘species barrier’. In the same study transgenic mice, which harbour the Syrian hamster prion gene and express hamster prion protein, challenged with the same scrapie infected hamster tissue all succumbed to disease with a relatively short incubation period of around 75 days (Scott et al., 1989). In human TSEs, transmission of sCJD into wild-type mice is notoriously difficult, with few if any mice developing clinical signs of disease. This is indicative of a substantial ‘species barrier’ between mouse and human (Collinge et al., 1995b; Bruce et al.,

1997; Hill et al., 1997). However, transgenic mice expressing only human PrP have been shown to be highly susceptible to sCJD, consistent with a lack of any ‘species barrier’ (Collinge et al., 1995b; Bishop et al., 2010).

However, investigations on the transmission of human TSEs supports the proposal that ‘transmission barrier’ rather than ‘species barrier’ may be a more appropriate term in describing the differences observed the efficiency of transmission of different TSEs. As described, sCJD is notoriously difficult to transmit to wild-type mice, whereas human transgenic mice are highly susceptible. In contrast, vCJD readily transmits to wild-type mice with an almost 100% attack rate, whereas transmissions to humanised transgenic mice are relatively less efficient than those observed with sCJD (Hill et al., 1997; Bishop et al., 2006). As the PrP amino acid sequence is the same in the donor PrP^{Sc} and in the recipient mice in these experiments, the differences observed in the efficiency of transmission cannot be accounted for solely by differences in species.

The experiments described above indicate that the ‘species barrier’ is also influenced by the TSE strain itself. This is further demonstrated with the BSE agent, which has successfully transmitted to a range of different species all with different PrP primary structures, either by natural transmission or experimentally (Bruce et al., 1994; Fraser et al., 1994; Bruce et al., 1997). Furthermore, the strain characteristics of the BSE agent are maintained on passage even through an intermediate species with a distinct PrP gene (Bruce et al., 1994).

1.9 Human TSEs

Human TSEs include CJD, kuru, Gerstmann-Sträusler-Scheinker (GSS) disease and fatal familial insomnia (FFI). Like scrapie, most human forms of TSEs have been shown to be transmissible to various animal species (Gajdusek et al., 1966; Gibbs, Jr. et al., 1968; Masters et al., 1981a; Collinge et al., 1995a; Bruce et al., 1997). Human TSEs are rare with an annual mortality rate of around 1- 1.5 cases per million of the population. As well as being rare, this group of diseases are unique in that they can occur in idiopathic, familial and acquired forms (Table 1.1). Kuru is a perfect example of the infectious, but non-contagious nature of TSEs. Kuru was spread through the Fore-speaking tribes of Papua New Guinea towards the end of the 1950s by ritualistic cannibalism, either through the ingestion of contaminated human tissues or transdermal infection through superficial wounds or skin scarification (Alpers 1995). Since the practice of cannibalism stopped the incidence of kuru has dropped dramatically. Occasional cases still occur suggesting extremely long incubation period which can exceed 50 years (Collinge et al., 2006).

GSS and FFI are examples of familial forms of human TSEs in which the cause of disease is attributed to the presence of an autosomal dominant mutation (including both point mutation and deletions and insertion mutations) of the *PRNP* gene. Familial forms of the disease are much less frequent and make up around 15% of human TSEs. The most common form of human TSE and the one which will be the main focus of this study is CJD. Specifically, this study focuses on the sporadic and variant forms of CJD. Therefore, the following sections will examine the clinical, neuropathological and biochemical features of CJD concentrating primarily on these two human TSEs

1.9.1 Creutzfeldt-Jakob Disease

CJD is the most frequently occurring form of human TSE. Spielmeyer introduced the term CJD in 1922 after combining the case reports of six patients reported by Hans-Gerhard Creutzfeldt (Creutzfeldt, 1920) and Alfons Jakob (Jakob, 1921). Retrospective studies on these six cases have since revealed that only two out of the six cases would meet the modern day criteria for CJD. CJD occurs worldwide and

can be classified into four main forms with respect to aetiology, clinical symptoms, neuropathology and more recently genetic and biochemical characteristics. The four types of CJD are described below.

1.9.1.1 Iatrogenic CJD

Less than 5% of CJD cases are classified as iatrogenic. Iatrogenic CJD results from the inadvertent human-to-human transmission of TSE infectivity in the course of medical procedures; including, via neurosurgical instruments (Will and Matthews, 1982), stereotactic electrodes (Bernoulli et al., 1977), tissue transmission such as corneal or dura mater grafts (Duffy et al., 1974; Heckmann et al., 1997; Brown et al., 2000) and also through the administration of human growth hormone treatment (Koch et al., 1985; Powell-Jackson et al., 1985). In such cases the patient has had direct exposure to the TSE agent by contact with brain tissues or extracts contaminated by the sCJD agent. More recently, the observation of five instances of vCJD infection in individuals following transfusion with blood or treatment with blood products from vCJD patients who were asymptomatic at time of donation, highlights the risk of the iatrogenic spread of vCJD (Llewelyn et al., 2004; Peden et al., 2004; Wroe et al., 2006; Health Protection Agency., 2009; Peden et al., 2010).

1.9.1.2 Familial CJD

Like GSS and FFI, familial CJD (fCJD) is a genetic disorder in which the cause of disease is attributed to an underlying mutation of the human *PRNP* gene. It is speculated that many of these mutations occur around the α -helical regions of the prion protein possibly reducing the structural stability of the protein, predisposing the host to the disease. Familial CJD cases account for around 10-15% of human TSEs although this figure is variable between different countries according to the presence of affected kindreds (Kovacs et al., 2005).

1.9.1.3 Sporadic CJD

Sporadic CJD accounts for the majority of human TSE cases (~ 85%) but still remains a relatively rare disorder with a world wide incidence of around 1 – 1.5 case per million of the population (Ladogana et al., 2005). Sporadic CJD is an idiopathic disorder in which cases occur in isolation with no evidence of an association with mutations in the human PrP gene or any epidemiological evidence for a direct exposure to the TSE agent as in the acquired forms. Current hypothesis regarding the aetiology of sCJD focuses on either an unidentified somatic mutation in the prion protein gene, the spontaneous conversion of PrP^C to PrP^{Sc} or an as yet unidentified environmental exposure to a TSE. Horizontal transmission from animals or humans has also been suggested, however no evidence has been put forward to support the hypothesis that this accounts for the vast majority of cases. A possible link between sCJD and general surgery has also been described; however, questions over recall bias in these findings can not be excluded (Collins et al., 1999; Ward et al., 2009).

Sporadic CJD has a median age at onset in the seventh decade and is rarely found in patients under 40 years old. However, several cases of sCJD have been reported world-wide in patients under the age of 20 (Monreal et al., 1981; Brown et al., 1985; Berman et al., 1988; Kulczycki et al., 1991; Murray et al., 2008). As has been discussed earlier in this chapter (section 1.3.1.1) there does appear to be a genetic susceptibility to sCJD. Analysis of the polymorphism at codon 129 on the *PRNP* gene has demonstrated that methionine homozygosity is a risk factor for the development of disease, with about 70% of cases having this genotype (Palmer et al., 1991; Alperovitch et al., 1999).

In sCJD, clinical manifestations can vary widely from case to case and are substantially affected by the genotype at codon 129 of the host prion protein gene (Table 1.4). Typically, sCJD presents with a rapidly progressive dementia often associated with cognitive disturbances such as confusion and memory loss. In addition, there is widespread neurological involvement including cerebellar ataxia and, most characteristically myoclonus. As the disease progresses, mental disturbances become more prominent until the disease culminates in a terminal

akinetic mute state. In around 40-60% of cases visual disturbances are often noted and in around three quarters of patients there is also an abnormal electroencephalogram (EEG) in which periodic sharp-wave complexes are observed. Raised cerebrospinal (CSF) levels of 14-3-3 protein, S-100 and neuronal specific enolase (NSE), although not specific for sCJD may be helpful diagnostically (Jimi et al., 1992; Zerr et al., 1995). Magnetic resonance imaging (MRI) can also be useful with some cases exhibiting a characteristic signal change in the putamen and caudate. The clinical duration in sCJD is usually short with around 70% of deaths usually within 6 months from first symptoms.

As with the clinical features, sCJD shows a great diversity in the neuropathology, specifically in the nature, severity and location of spongiform change within the brain, the presence or absence of amyloid plaques and in the patterns of PrP accumulation. In the case of spongiform change this can vary from focal regions of microvacuolation (Figure 1.1) to more extensive areas of confluent spongiform change, whilst in the most extreme cases status spongiosis has been reported (Masters and Richardson, Jr., 1978; Gambetti et al., 2003). The presence of amyloid plaques is only a feature of a small proportion of sCJD cases. In cases which do show amyloid plaque formation, these are generally confined to the cerebellar cortex (Figure 1.1). PrP deposition and immunoreactivity generally occurs in three main patterns in sCJD; plaque type, diffuse synaptic or granular and patchy/perivacuolar types in which much of the PrP labelling are found around vacuoles. As with the clinical features, these variations in neuropathological change are thought to be influenced by the *PRNP* codon 129 genotype of the individual (Table 1.4). In addition, the different biochemical PrP^{res} types identified in cases of sCJD are also thought to heavily influence the clinico-pathological phenotype of disease. The different PrP^{res} types associated with sCJD are discussed in detail later in this Chapter (Chapter 1, section 1.10).

Table 1.4: Proposed sub-classification of sCJD (adapted from Parchi et al., 1999)

sCJD subtype	Previous Classification	% of Cases	Duration (months)	Clinical Features	Neuropathological Features
MM1/MV1	Myoclonic, Heidenhain variants	70	3.9	Rapidly progressive dementia, early and prominent myoclonus, typical EEG; visual impairment or unilateral signs at onset in 40% of cases	“Classic CJD” distribution of pathology; often prominent involvement of occipital cortex; “synaptic type” PrP staining; in addition, one-third of cases shows confluent vacuoles and perivacuolar PrP staining
VV2	Ataxic variant	16	6.5	Ataxia at onset, late dementia, no typical EEG in most cases	Prominent involvement of subcortical, including brain stem nuclei; in neocortex, spongiosis is often limited to deep layers; PrP staining shows plaque-like, focal deposits, as well as prominent perineuronal staining
MV2	Kuru-plaques variant	9	17.1	Ataxia in addition to progressive dementia, no typical EEG, long duration (>2 yr) in some cases	Similar to VV2 but with presence of amyloid-kuru plaques in the cerebellum, and more consistent plaque-like, focal PrP deposits
MM2-thalamic	Thalamic variant	2	15.6	Insomnia and psychomotor hyperactivity in most cases, in addition to ataxia and cognitive impairment, no typical EEG	Prominent atrophy of the thalamus and inferior olive (no spongiosis) with little pathology in other areas; spongiosis may be absent or focal, and PrP ^{Sc} is detected in lower amount than in the other variants
MM2-cortical	Not established	2	15.7	Progressive dementia, no typical EEG	Large confluent vacuoles with perivacuolar PrP staining in all cortical layers; cerebellum is relatively spared
VV1	Not established	1	15.3	Progressive dementia, no typical EEG	Severe pathology in the cerebral cortex and striatum with sparing of brain stem nuclei and cerebellum; no large confluent vacuoles, and very faint synaptic PrP staining

PrP - prion protein

PrP^{Sc} – Protease-resistant prion protein

Due to the clinico-pathological heterogeneity observed in sCJD, attempts have been made to correlate these phenotypic variations. This has resulted in a proposed sub-classification system for sCJD describing six subtypes of sCJD, defined by their clinical and neuropathological phenotype (Table 1.4) (Parchi et al., 1999). These six phenotypic subtypes roughly correlated with the six possible combinations of *PRNP* codon 129 genotype and PrP^{res} type (MM1/MV1, MM2 cortical, MM2 thalamic, MV2, VV1 and VV2) and have been suggested to represent distinct sCJD strains of agent.

Since this study commenced, a novel form of human TSE has been identified. Initially termed protease-sensitive prionopathy (PSP^r), this human TSE was based on an original cohort of 11 patients all of which were homozygous for valine at codon 129 on the human PrP gene (Gambetti et al., 2008). The defining feature of these PSP^r cases was the unusual biochemical properties, in which the abnormal form of PrP was found to be much less resistant to protease digestion (Gambetti et al., 2008). Neuropathological features were also unusual, in particular the accumulation of microplaques in the brain which stained intensely on immunohistochemistry for the prion protein. Since the original description, a further 19 cases have been reported including patients who were homozygous for methionine and heterozygous for valine and methionine at codon 129 on the human PrP gene (Head et al., 2009; Jansen et al., 2010; Rodríguez-Martínez et al., 2010). The condition has since been renamed as variably protease-sensitive prionopathy (VPSP^r) to reflect the variable sensitivity in protease digestion reported between the three different codon 129 genotypes. VPSP^r cases can be classified as sporadic TSEs as there are no identifiable risk factors for exposure to a TSE and no mutations in the coding sequence of the prion protein gene have been found.

1.9.1.4 Variant CJD

Variant CJD was first identified in the UK in 1996 (Will et al., 1996). Initially referred to as ‘new variant’ CJD (nvCJD) and since shortened to vCJD, this form of the disease was described in patients of an uncharacteristically young age with a much longer disease duration than that typically observed in sCJD. There is now convincing evidence to support the initial hypothesis that vCJD is caused by human infection with the BSE agent, most probably through the consumption of contaminated meat products (Collinge et al., 1996; Lasmezas et al., 1996; Bruce et al., 1997; Hill et al., 1997; Scott et al., 1999). Variant CJD therefore represents an acquired form of human TSE, caused by a cross-species transmission from cattle to human. To date, over 170 cases of vCJD have been identified in the UK. Currently, all confirmed vCJD cases who have undergone genetic analysis have been shown to be homozygote for methionine at codon 129 of *PRNP* which clearly indicates a genetic susceptibility in this genotype (Bishop et al., 2009). However, recent findings suggest that the other *PRNP* codon 129 genotypes are also susceptible to infection with the BSE agent (Peden et al., 2004; Hilton et al., 2004b; Ironside et al., 2006; Peden et al., 2010). The incubation period in infected MV and VV individuals may be longer than that observed in the MM population (Bishop et al., 2006). Annual mortality rates now suggest that the vCJD outbreak is in decline in the UK following a peak in 1999/2000 (<http://www.cjd.ed.ac.uk/cjdq64jun10.pdf>). However, as fears over a greater primary epidemic subside, public health concerns over the possible secondary transmission of vCJD have heightened due to recent reports of person-to-person transmission of vCJD infectivity by blood transfusion and blood products (Llewelyn et al., 2004; Peden et al., 2004; Wroe et al., 2006; Health Protection Agency., 2009; Peden et al., 2010).

The clinical phenotype of vCJD differs from that of sCJD and all other human TSEs. The unusually young age at which vCJD occurs is in contrast to all other forms of the disease (mean age at death of 29 years) and in comparison with sCJD, vCJD has a much longer disease duration (mean survival is 14 months). The clinical symptoms in vCJD are relatively uniform between cases. Patients present primarily with psychiatric and behavioural disturbances, anxiety, depression, and withdrawal are

often accompanied by sensory abnormalities. This is usually followed by ataxia, myoclonus, variable movement disorders and dementia. The later stages are similar to sCJD, with terminal akinetic mutism in many cases. Examination of MRI scans in vCJD has shown that there is often a high signal in the posterior thalamus the “pulvinar sign”, on T2 in around 90% of cases, more than likely a reflection of the extensive astocytic proliferation in this brain region in vCJD cases. The EEG shows no periodic complexes and CSF 14-3-3 assay is positive in only 50% of cases (Will et al., 1996; Will et al., 2000; Green et al., 2001; Knight and Will, 2004).

In contrast to the marked heterogeneity in the neuropathological features of sCJD cases, vCJD have proved highly stereotypic. Variant CJD has a distinct pathology in terms of plaque accumulation. The presence of multiple ‘florid plaques’ within the cerebral cortex and cerebellum is the most striking feature of this disease (Will et al., 1996; Ironside, 1996; Ironside et al., 2000). These plaques have the appearance of the classic kuru-type plaque and are surrounded by a halo of spongiform change. These plaques were first described in Icelandic scrapie and have also been describe in mice infected with the 11A scrapie strain (McBride et al., 1988) and have been reported in BSE-inoculated primates (Lasmezas et al., 1996). Florid plaques are rarely observed in human TSEs other than vCJD although occasional florid plaques have been reported in cases of iatrogenic CJD in patients who have received a dura mater graft (Takashima et al., 1997; Shimizu et al., 1999; Kretzschmar et al., 2003). In addition to florid plaques in vCJD there is also the accumulation of smaller plaque like aggregates usually found in clusters in the cerebral cortex. As well as plaques lighter, ‘feathery’ deposits of PrP are found surrounding the neurons or blood vessels within the cerebellar and cerebral cortex.

Variant CJD differs from other forms of human TSE in that there is a widespread, abundant and readily detectable accumulation of PrP^{res} in lymphoid tissues throughout the body. This is best observed in spleen, tonsil, appendix and lymph nodes (Hill and Collinge, 2002; Joiner et al., 2002; Head et al., 2004a). The significance of PrP^{res} within peripheral tissues in vCJD is demonstrated by the inclusion of a positive tonsil biopsy becoming part of the diagnostic criteria for

vCJD. More recently, with the increased sensitivity in detection assays, the detection of PrP^{res} in tissues other than those of the lymphoreticular system indicates that the number of tissues and organs showing the presence of PrP^{Sc} in vCJD may be greater than first thought (Glatzel et al., 2003; Haik et al., 2003; Head et al., 2004b; Peden et al., 2006; Peden et al., 2007; Notari et al., 2010). The detection of infectivity outside the CNS (Bruce et al., 2001) also has important consequences for the spread of vCJD by iatrogenic means, particularly in regard to the use of surgical instruments. Some of the important distinctions between vCJD and sCJD in terms of clinical and neuropathological features are listed in Table 1.5.

Table 1.5: Clinical and neuropathological features of sporadic and variant CJD

	sporadic CJD	variant CJD
Mean age at death	66years	29 years
Median duration of illness	4 months	14 months
Thalamic MRI high signal	Caudate /putamen (60%)	Pulvinar (90%)
EEG	‘Typical’ 70%	‘Typical’ 0%
Presence of plaques	Kuru type 10%	Florid plaques 100%
PrP ^{res} distribution	Diffuse/synaptic Patchy/peri-vacuolar	Plaque like clusters Peri-neuronal
PrP ^{res} in non CNS tissues	reported in muscle (Glatzel et al., 2003; Peden et al., 2006), spleen (Glatzel et al., 2003), pituitary gland (Peden et al., 2007)	Reported in lymphoid tissue, peripheral nervous system (Haik et al., 2003; Head et al., 2004b),pituitary gland (Peden et al., 2007),skeletal muscle (Peden et al., 2007), dura mater, liver, pancreas, kidney, ovary, uterus and skin (Notari et al., 2010)

1.10. Biochemical aspects of human TSEs

The first demonstration that different PrP^{res} types are associated with different disease phenotypes in human TSEs was in a study investigating the basis for the clinico-phenotypic heterogeneity observed in cases of FFI and fCJD, two familial TSEs which have an identical mutation at codon 178 on the human *PRNP* gene (Monari et al., 1994). Biochemical analysis of CNS tissue by Western blotting showed differences in the extent of N-terminal truncation between these two TSEs resulting in two major size classes; one associated with FFI resulting in a ~19kDa unglycosylated fragment and a second associated with fCJD resulted in a ~21kDa fragment. FFI and fCJD differ in *PRNP* codon 129 genotype; the presence of methionine at codon 129 encodes FFI whereas the presence of valine encodes fCJD. Therefore, it was proposed that the combination of the mutation at codon 178 on the *PRNP* gene and the codon 129 *PRNP* genotype, determines the clinico-pathological phenotype by producing two different PrP^{res} types (Monari et al., 1994). The suggestion that different PrP^{res} types can have an influential role in determining disease phenotype has since been supported by studies of sCJD where an association between disease phenotype and PrP^{res} type has been observed (Parchi et al., 1999).

1.10.1 Molecular classification of CJD

Two competing classification systems have been proposed for CJD as a result of differences found in the mobility of protease resistant PrP after Western blot and the relative abundance of the different glycosylated PrP^{res} isoforms (referred to as the glycoform ratio). The two classification systems are those originally outlined by Collinge and co-workers (Collinge et al., 1996; Wadsworth et al., 1999a; Wadsworth et al., 1999b; Hill et al., 2003) and those of Parchi and Gambetti (Parchi et al., 1996; Parchi et al., 1997; Parchi et al., 1999). In addition to the mobility of PrP^{res} and glycoform ratio (PrP^{res} type), both systems include patient results from the *PRNP* genotype at codon 129 as an additional classification method, as the M/V polymorphism at this site has been implicated as playing an important role in the phenotypic variability in CJD (Alperovitch et al., 1999). The following section will outline these two classification systems and show chronologically how the complexities in 'molecular strain typing' have arisen.

1.10.1.1 Collinge classification system

(Collinge et al., 1996)

In their first publication on molecular strain typing of human TSE, Collinge and co-workers examined the physico-chemical properties of PrP^{Sc} in sporadic, iatrogenic and variant cases of CJD. Western blot results identified four distinct patterns of PrP^{res} that were termed types 1, 2, 3 and 4, associated with sporadic and acquired human TSEs (Figure 1.7a). Two distinct PrP^{res} types were reported in sCJD cases. These two PrP^{res} types, designated type 1 and type 2 resulted in PrP fragments of differing molecular mass. The PrP^{res} glycoform ratio showed subtle differences between these two sCJD PrP^{Sc} types, with type 2 showing a greater abundance in the monoglycosylated fragment of the protein. By incorporating the codon 129 genotype of each case examined, Collinge and co-workers found that type 1 PrP^{res} is uniquely associated with methionine homozygosity (MM) at *PRNP* codon 129 whereas, type 2 PrP^{res} is associated with a minority of MM patients as well as all the valine homozygotes (VV) and methionine/valine (MV) heterozygotes. Type 3 PrP^{res} was found in VV and MV cases of iatrogenic CJD, which had arisen from peripheral routes of exposure to TSEs, in particular patients who had received pituitary growth hormone. Type 3 was characterised by a downward shift in all three bands consistent with a decrease in the molecular mass of around 2-3kDa when compared to type 2 (Figure 1.7a). The fourth pattern of PrP^{res}, type 4, was associated with all vCJD cases. Type 4 resulted in a ~19kDa fragment size, similar to that observed in type 2 and type 3 cases but were distinguished by the predominance of the diglycosylated fragment (Figure 1.7a). All type 4 cases were associated with methionine homozygosity at *PRNP* codon 129. The identification of this vCJD molecular signature was an important step in providing molecular evidence that vCJD represented a new and distinct human TSE strain.

(Wadsworth et al., 1999a)

The Collinge classification system was modified in 1999 with a publication by Wadsworth and co-workers demonstrating that strain-specific protein conformations in human TSE may be influenced by metal binding to PrP^{Sc}. Wadsworth examined sCJD (both type 1 and type 2) cases homozygous for methionine at codon 129. They

found that treatment of these cases with 20mM of the metal ion chelator EDTA prior to proteinase K treatment, changed the pattern of cleavage and instead of producing their own distinct patterns, produced an indistinguishable banding pattern. The resulting PrP^{res} fragments had a smaller molecular mass than either type 1 or type 2 and were termed type 2⁻ (Figure 1.7c). No modification of type 2 and type 4 was observed after treatment with EDTA.

(Wadsworth et al., 1999b)

In a slightly later publication, Wadsworth observed that type 3 PrP^{res}, previously restricted to cases of iatrogenic CJD, was now found to include cases of sCJD with a codon 129 genotype of either MV or VV (Figure 1.7c). This now allowed for three possible PrP^{res} types in sCJD as classified by the mobility of PrP^{res} and glycoform ratio on Western blot as well as the genotype at codon 129 of *PRNP*.

(Hill et al., 2003)

In this large scale study, Hill *et al.*, used a modified Western blotting technique to confirm that different PrP^{res} types are associated with cases of sporadic, iatrogenic and variant CJD. However, in addition to the four types described previously (Collinge et al., 1996), a novel PrP^{res} type, termed type 6, was reported in a single case of sCJD in a methionine homozygote patient. Type 6 is characterised on PrP fragment size alone, whereby the relative molecular mass of these fragments are larger than those observed in type 1 and type 2 cases (Figure 1.7d). In addition, the classification of PrP^{res} type 3 was modified with the addition of a single type 3 PrP^{res} in an MM patient. Further analysis of the glycoform ratios in type 3 cases also showed that MV type 3 cases could be further distinguished by a more predominant unglycosylated fragment, a feature not previously reported (Figure 1.7d). The modifications made in the Western blot protocol also identified consistent differences in the fragment size between PrP^{res} type 3 and type 4 cases. Type 4 now showed a slightly larger molecular mass when compared with type 3 (Figure 1.7d).

In his 1999 publication in *Nature Cell Biology* (Wadsworth et al., 1999a), Wadsworth observed that PrP^{res} type 1 and type 2 (MM cases only) showed a slight

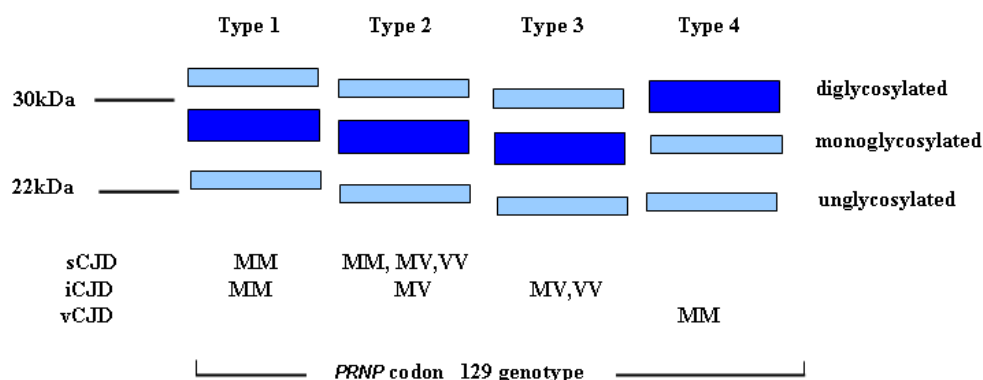
shift in fragment size following chelation of metal ions with EDTA prior to digestion with proteinase K resulting in indistinguishable pattern of digestion products designated type 2⁻ (Figure 1.7c). A similar finding was reported in this study in a single PrP^{res} type 2 from an MV sCJD case and the single type 6 using the same protocol. The shift in fragment size was also observed with type 3 cases but only in the valine homzygotes.

A schematic representation summarising the results and the PrP^{res} types described in the four publications above, representing the Collinge classification system for human TSEs are shown in Figure 1.7.

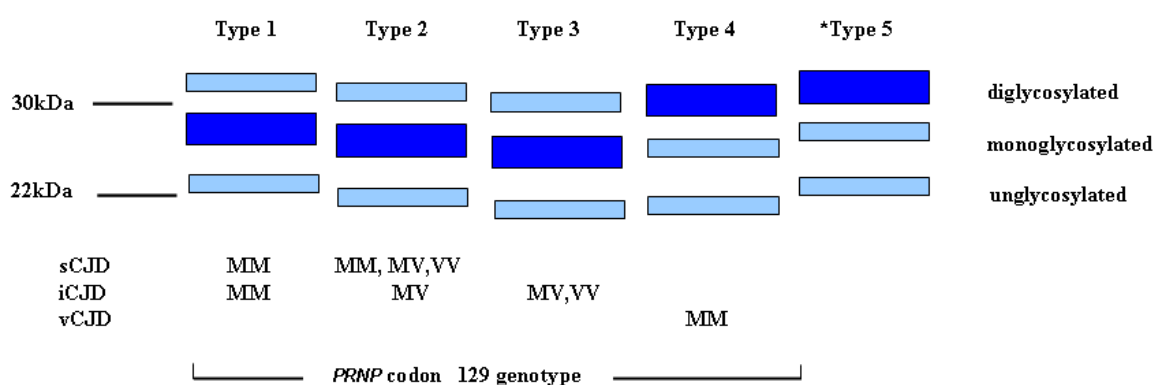
Figure 1.7: Collinge's molecular classification of human TSEs

Schematic representation of the development of the molecular classification system for human TSEs as reported by Collinge and co-workers. The illustrations show PrP^{res} types 1-6 and their relationship with the *PRNP* codon 129 genotype.

(a) Collinge et al., 1996

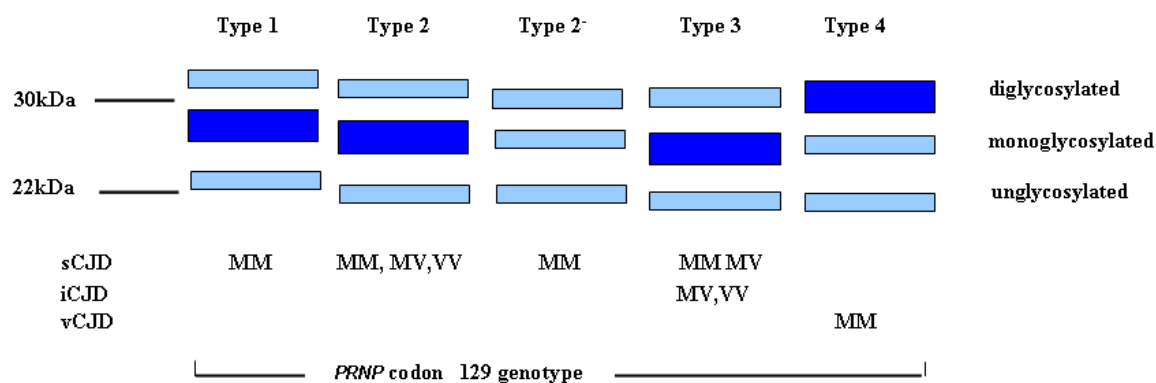


(b) Hill et al., 1997

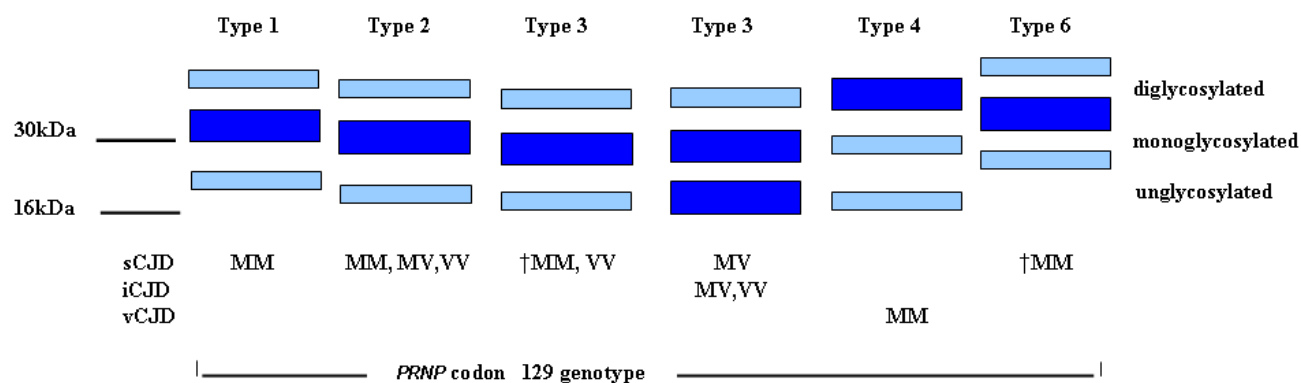


*Type 5 transmission of variant CJD to *HuPrP129V/+Prn-p0/0* transgenic mice

(c) Wadsworth et al., 1999a, Wadsworth et al., 1999b



(d) Hill et al., 2003



† Single cases observed with the respective PrP^{Sc} types

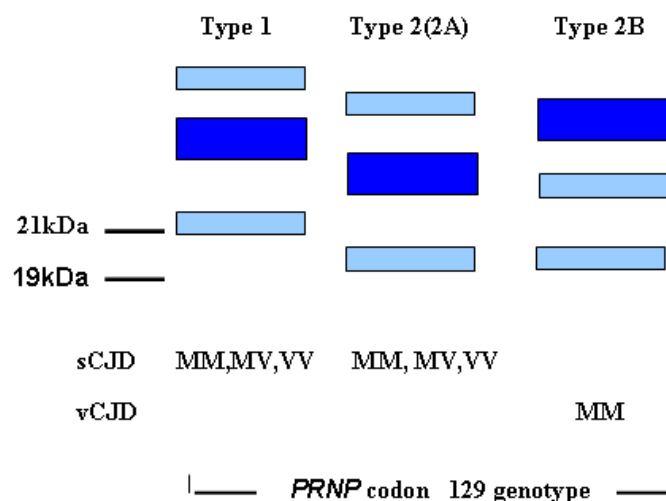
1.10.1.2 Parchi & Gambetti classification system

Similar studies carried out by Piero Parchi and Pierluigi Gambetti reported only two PrP^{res} types associated with sCJD cases. These two sCJD PrP^{res} types differed in the migration of the unglycosylated fragment of the protein and in the ratio of the three PrP glycoforms. Type 1 was characterised by a relative molecular weight of the unglycosylated fragment of ~21kDa with type 2 characterised by a molecular weight of ~19kDa (Parchi et al., 1996; Parchi et al., 1997; Parchi et al., 1999). At further variance with Collinge's classification system, Parchi and Gambetti reported that PrP^{res} type 1 and type 2 were detected in sCJD patients from all three *PRNP* codon 129 genotypes, resulting in six possible subgroups of sCJD as classified by PrP^{res} type/*PRNP* codon 129 genotype combinations (Figure 1.8). These six subgroups of sCJD were found to show a close correlation to the six major subtypes of sCJD as classified according to the clinico-pathological phenotype of sCJD (Table 1.4) (Parchi et al., 1996; Parchi et al., 1999). This suggested that the PrP^{res} type/*PRNP* codon 129 genotype combinations may underlie the phenotypic variation in sCJD and indeed represent the molecular basis of sCJD strains. The most common sCJD subtype and representing around 70% of all sCJD cases were found to have a type 1 banding pattern and either a MM or MV *PRNP* codon 129 genotype. Cases homozygous for valine, with a type 1 banding pattern were the least frequent subtype of sCJD representing only 1% of all cases (Parchi et al., 1999).

In agreement with Collinge's classification system, Parchi and Gambetti identified only a single PrP^{res} type associated with vCJD cases. PrP^{res} associated with vCJD had an indistinguishable migration of the unglycosylated to that observed in type 2 sCJD cases resulting in a ~19kDa fragment. However, vCJD PrP^{res} could be distinguished from sCJD by the predominance in the diglycosylated form of the protein. Variant CJD PrP^{res} was designated type 2B to distinguish it from sCJD type 2 cases (Figure 1.8). Therefore, sCJD type 2 cases, where the monoglycosylated band predominates are often referred to as type 2A.

Figure 1.8: Parchi and Gambetti's molecular classification of human TSEs

Schematic representation of PrP^{res} types 1-2 and their relationship with *PRNP* codon 129.



Comparisons drawn between these two studies argued that the type 1 PrP^{res} reported by Parchi and Gambetti correspond to type 1 and type 2 described by Collinge and co-workers, with Collinge's type 3 corresponding to Parchi and Gambetti's type 2 (Parchi et al., 1997). In their original study, Parchi and Gambetti identified a number of cases with a small variation in the molecular mass of PrP^{res} type 1. They proposed that Collinge's type 1 and type 2 represented artefactual separation of such cases. This proposal was disputed by Collinge and co-workers who proposed that the differences are more than likely due to methodological differences between the two studies, in particular treatment with the metal ion chelator EDTA prior to proteinase K treatment, which was found to modify the PrP^{Sc} conformation resulting in faster migration of PrP^{res} (Wadsworth et al., 1999a). Further studies investigating the discrepancies between these two classifications suggest that the variation observed in N-terminal truncation may be due to the effects of pH variation during the preparations of tissues for analysis (Notari et al., 2004; Cali et al., 2006). Regardless of the differences between the Collinge classification and the Parchi and Gambetti sub-classification of sCJD, both are in agreement that the phenotypic heterogeneity observed within sCJD is dependant on the codon 129 *PRNP* genotype and the PrP^{res} type of the patient. Nomenclature apart the only substantial difference between the two classification systems in terms of sCJD sub-classification is the existence of two different phenotypes associated with Parchi and Gambetti MM1, termed 1MM and 2MM by Collinge. Whatever the basis of the difference between the two sets of results, a World Health Organisation (WHO) unpublished ring trial concluded that the Parchi and Gambetti sub-classification system was a more reproducible and transferable system (Mark Head, personal communication). The investigations of human TSEs described in this thesis follow the Parchi and Gambetti classification system for sCJD.

1.10.2 Current concerns relating to molecular strain typing

Biochemical characterisation of PrP^{res} as a means of strain typing and diagnosing CJD has come under scrutiny recently following reports that regional variation in PrP^{res} type occurs within cases of sCJD (Parchi et al., 1999; Puoti et al., 1999). This phenomenon was first reported in Parchi's original analysis of 300 sCJD cases, in which around 5% of cases examined demonstrated both PrP^{res} type 1 and type 2 within the cerebral cortex of the same brain sample (Parchi et al., 1999). Recent studies have confirmed these findings and indicated that the existence of mixed PrP^{res} types or 'co-occurrence' in the brain may be prevalent in a much higher proportion of cases (~ 30%) than first reported (Puoti et al., 1999; Schoch et al., 2006; Uro-Coste et al., 2008). Correct identification of these cases may be limited by the extent of tissue sampling and the sensitivity of the assay in detecting minority PrP^{res} components (Head et al., 2004a; Yull et al., 2006). It has also been suggested by two studies using antibodies raised to specifically recognise PrP^{res} type 1, that all cases of sCJD type 2 contain a proportion of PrP^{res} type 1 component (Polymenidou et al., 2005; Yull et al., 2009). The phenomenon of mixed PrP^{res} types may be an indication that the different PrP^{Sc} types can either interconvert or occur spontaneously. In either case this challenges or at the very least complicates the relationship between disease phenotype, PrP^{res} type, *PRNP* codon 129 genotype and agent strain. In addition to the extent of N-terminal truncation (electrophoretic mobility), glycosylation ratio is also proposed to show strain-specific variation. Recent reports that PrP^{res} glycoform profile also shows regional variation in sCJD cases further highlights the difficulties associated with PrP^{res} typing as a means of correctly identifying different human TSE and in providing a biochemical basis for agent strain (Levavasseur et al., 2008).

The phenomenon of mixed PrP^{res} types within sCJD cases has recently prompted the re-evaluation of the Parchi and Gambetti classification system for sCJD in which six sCJD subtypes have been proposed based on the correlation of clinical and pathological features of disease with variations in *PRNP* codon 129 and PrP^{res} type (Parchi et al., 1999). The main aim of this re-evaluation was to establish whether the presence of mixed PrP^{res} types in the brain results in a further distinct clinico-

pathological phenotype for inclusion in the current sub-classification system for sCJD. The first publication by Cali *et al.*, re-analysed 34 cases of sCJD in patients homozygous for methionine at *PRNP* codon 129 (13 MM1, 9 MM2 and 12 MM1+2 cases as previously classified based on Western blot analysis). Analysis of clinical, neuropathological and biochemical characteristics all indicated that sCJDMM1+2 do represent a unique sCJD subtype and should be included in the current sub-classification system (Cali *et al.*, 2009). In a slightly later publication, further re-analysis of all sCJD cases irrespective of genotype supported the inclusion of sCJDVV1+2 and sCJDMV1+2 as well sCJDMM1+2 as distinct subgroups within the classification system having shown unique neuropathological and biochemical phenotypes (Parchi *et al.*, 2009).

The publication of a case of CJD in a young Dutch patient also questioned the reliability of PrP^{res} isotyping as a diagnostic tool for the sub-classification of sCJD and as a means of characterising agent strain (Head *et al.*, 2001). The case in question focused on a 42 year old Dutch woman who was admitted to hospital with suspected CJD. A brain biopsy (right frontal cortex) was taken from this patient due to the young age at onset and atypical clinical symptoms. Neuropathological examination of the biopsy material demonstrated classic signs of sCJD with spongiform change, astrogliosis and neuronal loss. Western blot analysis using Parchi and Gambetti's classification system confirmed the diagnosis of sCJD and a type 1 PrP^{res} type with genetic analysis confirming a valine homozygote at codon 129 of *PRNP*. The patient died 18 months after onset of clinical symptoms, unusually long disease duration for sCJD. Permission for examination of the brain was provided. Histological examination confirmed the previous diagnosis on the biopsy material of sCJD. However, Western blot analysis on a sample from the left frontal cortex showed contrasting results to those of the biopsy with unglycosylated PrP^{res} type 2 mobility and a glycoform ratio that resembled vCJD not sCJD. The final diagnosis of this patient was confirmed as an atypical case of sCJD. This case highlighted the difficulties in interpreting PrP^{res} isotyping and the caution that must be taken when making a diagnosis. Again, the problems of strain typing in CJD were

raised with the question of conversion of PrP^{res} types during disease progression and the possibility of regional variation in PrP^{res} types.

1.11 Experimental transmission of CJD

If strain-specific information is enciphered within the different conformational variants of PrP^{Sc}, the biochemical properties of PrP^{Sc} would be expected to be conserved on transmission. Non-human primates have successfully been used in a number of experimental transmissions involving a wide range of human TSEs and were instrumental in the inclusion of kuru, sCJD and vCJD as human TSEs (Gajdusek et al., 1966; Gibbs, Jr. et al., 1968; Lasmezas et al., 1996). As primates are evolutionary closer to humans it is thought that they may represent the most accurate model for the pathogenesis of disease. In one of the largest TSE transmission studies, investigating the experimental transmission of brain homogenate from 300 human TSE cases in different primate species, the overall success rate for transmission was over 90 % for sCJD, iCJD and kuru with a lower rate of ~68% for the familial forms of the disease (Brown et al., 1994). Subsequently, comparison of the transmission characteristics of vCJD and BSE in macaques was to provide early support for the initial hypothesis that vCJD is BSE in humans (Lasmezas et al., 1996). Neither of these studies investigated the biochemical phenotype or the relationship of *PRNP* codon 129 genotype between the donor and the recipient. However, a later study investigating the experimental transmission of brain homogenate from sCJD and vCJD in the squirrel monkey, indicated that the biochemical phenotype is conserved on transmission to primates (Williams et al., 2007). However, in the case of sCJD, the findings of this study suggested that the resulting PrP^{res} type may be attributed to variation in the *PRNP* codon 129 genotype of the host. In addition to primate models, the bank vole (*Clethrionomys glareolus*) has also proved to be highly susceptible to human TSEs (Nonno et al., 2006). Successful transmission of brain isolates from some sCJD subtypes in the bank vole confirmed that PrP^{res} type is maintained on transmission in this animal model. These transmissions also suggest that the successful transmission from different sCJD subgroups may be influenced by the codon 129 genotype and PrP^{res} type of the tissue donor.

1.11.1 Mouse models of human TSE

Experimental transmission of human TSEs in wild-type mice has provided variable results. Sporadic CJD transmits inefficiently, often resulting in the absence of any clinical signs of disease, whereas vCJD transmission is highly efficient in wild-type mice, resulting in 100% of animals showing clinical signs of disease (Bruce et al., 1997). In order to overcome the 'transmission barrier' observed with sCJD, the majority of transmission studies involving human TSEs now concentrate on the use of transgenic mice expressing human PrP.

There are two main methods for the generation of transgenic mice; random genetic insertion (RGI) and gene targeting (GT). The most common transgenic mice associated with human TSE transmission are those generated by RGI which are produced by the injection of cloned PrP genes into fertilised mouse oocytes. Using this method of generation, there is no control over the insertion point or the number of copies of the PrP gene that have been generated. These mice often result in the over-expression of the PrP gene. There may also be a loss in function if the inserted PrP gene disrupts other genetic components. The second method of GT, involves the specific replacement of the *Prn-p* gene with the genetic material for insertion, in the case of human transgenic mice, insertion of the human *PRNP* gene. The benefits of these mice are that the inserted human *PRNP* gene remains under the correct transcriptional control in the mouse.

Experimental transmission of human TSEs by Collinge and co-workers have provided experimental evidence to support the use of PrP^{res} typing as a reliable and consistent method of strain typing. The use of transgenic mice expressing only human PrP has been an important step in transmission studies, allowing direct comparisons of the PrP protein. In his first study on molecular strain typing of CJD, Collinge demonstrated the successful transmission of his type 2 and type 3 PrP^{res} to Tg152 mice, RGI generated transgenic mice expressing human PrP not murine PrP and encoding for valine at codon 129 (designated HuPrP129V^{+/+} *Prn-p*^{0/0}) (Collinge et al., 1996). This showed not only the lack of a 'species barrier' but importantly for the prion hypothesis and the proposal that strain variation is encoded by different PrP

conformations, that after transmission these two sporadic and iatrogenic types (type 1-3) retained the biochemical characteristics of the individual inoculum (Collinge et al., 1996). However, transmission of type 1 CJD resulted consistently in a type 2 banding pattern of human PrP^{res}, perhaps a direct result of the mis-match of genotype at codon 129 on *PRNP*. Transmission of types 1, 2 and 3 through wild type mice (FVB and C57BL) were less successful with only occasional transmissions with much longer incubation periods consistent with the presence of a 'species barrier'. Crucially the glycoform ratios observed in these transmissions were identical to the three types of CJD.

In a follow up study, transmission of vCJD to Tg152 mice proved less efficient when compared with sporadic and iatrogenic CJD and in the cases that did transmit, the incubation period was much longer (Hill et al., 1997). In FVB mice, transmission of vCJD proved much more efficient than sporadic and iatrogenic CJD although incubation period was more variable and longer. Transmission of BSE in FVB and Tg152 mice showed similar results to that of vCJD with efficient transmission into FVB mice but less efficient transmission in the transgenics. When glycoform patterns were examined, vCJD and BSE transmissions to FVB mice were indistinguishable, producing a type 4 pattern consistent with the inoculum. However, in the vCJD transmissions to Tg152 mice, a similar glycoform ratio to type 4 was observed but the fragment sizes differed to that of the inoculum. This pattern was given a new PrP^{res} typing, designated type 5 (Figure 1.7b), and thought to represent a prion strain switch resulting from mis-match of the codon 129 polymorphism between inoculum and host human PrP (Hill et al., 1997). Western Blotting of the primary BSE transmissions in Tg152 mice did not demonstrate the presence of PrP^{res}, possibly due to lower titres of the agent present in the BSE inoculum when compared to vCJD.

In order to establish if the poor transmission of vCJD into Tg152 mice was indeed a result of the mis-match at codon 129 of *PRNP*, inoculum used in previous transmission studies (Collinge et al., 1996; Hill et al., 1997) was transmitted to Tg35 mice, RGI generated transgenic mice expressing human PrP not murine PrP and

encoding methionine at codon 129 (HuPrP129M^{+/+}, Prn-p^{0/0}). Surprisingly, Tg35 mice showed even more resistance to vCJD than had been found with Tg152 mice as judged by the development of clinical disease (Asante et al., 2002). However, investigation of the mice for subclinical infection provided very different results. Pathological examination of Tg35 mice challenged with vCJD provided evidence of prion infection in 100% of the challenged mice with the presence of PrP plaques many of which were of the 'florid' type. In terms of biochemical analysis, Western blotting showed a type 4 PrP^{res} pattern characteristic of vCJD, consistent with the prion hypothesis that prion strain type is encoded by the biochemical properties of PrP^{res} and, that the *PRNP* codon 129 polymorphism plays a key role. Re-examination of the vCJD transmissions in Tg152 mice (Collinge et al., 1996; Hill et al., 1997), which were negative on clinical assessment, showed no evidence of subclinical disease. In transmissions of sCJD to Tg35 mice the codon 129 genotype of the inoculum played an important role. Tg35 mice were highly susceptible to prions from sCJD *PRNP* 129 MM patients, but less susceptible to those from sCJD *PRNP* 129 VV patients. Transmission of sCJD of the MV *PRNP* genotype was associated with either consistent short duration characteristics as with MM or long disease duration as observed with VV cases (Asante et al., 2002).

Transmission of BSE into Tg35 mice also resulted in both clinical disease and subclinical infection (Asante et al., 2002). However, only a single mouse showed the characteristic PrP^{res} type 4 after Western blotting with the neuropathological features characteristic of vCJD inoculated mice. In the remaining mice an alternative phenotype was observed, with a distinctive human PrP^{res} type produced on Western blot showing a different fragment size of unglycosylated PrP^{res}. The PrP^{res} glycoform ratio of these mice was also very different from type 4 PrP^{res} with the monoglycosylated portion the most abundant. Comparison with Collinge's known human types indicated that this type corresponded to the type 2 PrP^{res} observed in sCJD and iatrogenic cases. These cases also showed a shift in fragment size to type 2⁻ PrP^{res} after EDTA treatment prior to proteinase K digestion, a feature that had not been observed with type 4 PrP^{res} cases. Neuropathological examination confirmed subclinical disease in these mice but showed no specific immunoreactivity; in

particular, there were no florid or other plaques. The authors raised the possibility that some humans infected with the BSE agent may develop a clinical disease indistinguishable from sCJD associated with type 2 PrP^{res}. These transmissions were repeated in a second transgenic line expressing HuPrPM129, generated as described for Tg35 but which have a level of expression of human PrP four fold higher than a normal pooled human brain standard. Transmission of vCJD to these mice mirrored vCJD transmissions in Tg35 mice but showed no signs of the alternate type 2 PrP^{res} pattern that was obtained with the Tg35 mice. Western blot and neuropathological examination of these mice showed a phenotype consistent with vCJD.

More recently, a series of transmission experiments by the group lead by Jean Manson has provided valuable information on susceptibility to human TSEs and in characterising the agent strain in vCJD and sCJD. The transgenic models used in this series of experiments were produced using gene targeting in which the mouse PrP gene was directly replaced by the human PrP gene with the codon 129 MM, MV and VV genotypes. In their first series of experiments, successful transmission of vCJD, as determined by the appearance of clinical and pathological signs of a TSE was observed in all three transgenic mouse lines (HuMM, HuMV and HuVV), evidence that all *PRNP* codon 129 genotypes are susceptible to secondary transmission of vCJD (Bishop et al., 2006). However, differences in the appearance of clinical signs and in the progression of pathological features of TSE in the different transgenic lines indicated that there is a graduation of incubation period from MM to MV to VV. This was proposed to indicate that vCJD in an MV or VV genotype may be subject to a lengthy preclinical phase. Furthermore, differences observed in the pattern of PrP accumulation in the different mouse lines raised the possibility that vCJD in these other genotypes could result in a disease phenotype distinct from that recognised in vCJD in MM individuals (Bishop et al., 2006).

In their follow up study, Bishop and co-workers further investigated the vCJD agent strain in transgenic and wild-type mice to determine whether there was any modification to the vCJD strain after secondary human transmission of vCJD. In this study, inoculation of brain homogenate from the first case of transfusion associated

vCJD showed only minor differences in biological properties from vCJD derived from BSE which was previously reported in these mouse lines (Bishop et al., 2008). Reassuringly, the similarities observed in the transmission properties of primary and secondary vCJD cases in transgenic and wild-type mice were consistent with the proposal that there are no major changes in the agent strain after secondary transmission via blood transfusion.

In their most recent publication, all three transgenic mouse lines (HuMM, HuMV and HuVV) were challenged with the six different (Parchi and Gambetti) sCJD subgroups, as classified by *PRNP* codon 129 genotype and PrP^{res} type, in order to define the agent strain in sCJD and the influence of codon 129 genotype on the transmission properties of sCJD (Bishop, 2008; Bishop et al., 2010). In summary, analysis of clinical, pathological and biochemical properties in the mice suggested that the six sCJD subgroups behave as four distinct strains of agent (MM1 and MV1; MV2 and VV2; VV1; MM2) on transmission in these transgenic mouse models. This study is closely related to the work presented in this thesis examining the agent strain of sCJD on transmission to wild-type mice. Therefore, the results of this study will be directly compared and discussed in Chapter 7 (section 7.2.1.1) in this thesis.

1.12 Thesis aims

The working hypothesis of this thesis was that the different disease phenotypes of sCJD represented different strains of agent. However, there was no evidence to support this proposition and no rigorous studies had been reported. The aim of this thesis is to analyse existing mouse transmission studies to investigate the relationship between disease phenotype, *PRNP* codon 129 genotype, PrP^{res} type and agent strain. In extending these mouse transmissions, this thesis also aims to investigate the presence of infectivity in tissues other than brain in CJD patients. In addressing these aims it will ask a number of specific questions.

- 1. Do the six different sCJD subgroups, as classified by *PRNP* codon 129 and PrP^{res} type, behave as different strains of agent on transmission to wild-type mice? This will consist of:**
 - i) Confirming the PrP^{res} type of brain inocula used in the transmission series.
 - ii) Characterising the sCJD agent using the classical strain typing parameters of incubation period and lesion profile to establish if the different sCJD subgroup show unique transmission properties in the mice.
 - iii) Establishing whether the biochemical phenotype (PrP^{res} type) is conserved on transmission consistent with that which would be predicted from the prion hypothesis.
- 2. How do transmissions from sCJD, proposed to have more than one strain of agent compare with vCJD, believed to be a single strain of agent? This will consist of:**
 - i) Analysing the transmission properties of brain inocula from 10 vCJD patients in order to establish whether there are similarities, consistent with the proposal that vCJD is caused by a single strain of agent.
 - ii) Comparing the transmission properties of vCJD with that of all six sCJD subgroups to determine whether there is any relationship between the vCJD agent and that of any sCJD subgroup.

- 3. Can the detection of infectivity in vCJD patients be extended to tissues other than brain, spleen and tonsil? Furthermore, can infectivity be detected outside the CNS in sCJD? This will consist of:**
- i) Establishing any evidence of positive transmission of a TSE (as determined by clinical signs of disease, lesion profile and PrP deposition) from a wide-range of lymphoid and peripheral neural tissues from vCJD patients on transmission to wild-type mice, extending the evidence of peripheral infectivity in vCJD.
 - ii) Determining evidence of positive transmission from sCJD spleen in order to establish evidence of a peripheral involvement in sCJD.
 - iii) To establish whether infectivity can be detected in different vCJD and sCJD blood fractions in wild-type mice.
- 4. Is agent strain subject to any tissue specific modifications? This will consist of:**
- i) Characterising the agent strain in lymphoid tissues, peripheral neural tissues and biological fluids showing positive transmission to mice.
 - ii) Comparison of the transmission properties of vCJD/sCJD brain inocula with those of other tissues and fluids from vCJD/sCJD patients to determine whether the agent strain is subject to any tissue-specific modifications.

Chapter 2: Materials and Methods

The series of experiments described in this thesis involves a large scale study investigating the transmission properties of sCJD and vCJD in wild-type mice. This is part of an on-going collaboration between the National CJD Research & Surveillance Unit (NCJDRSU), Edinburgh University and the Neuropathogenesis Division (NPD), Roslin Institute (formerly part of the Neuropathogenesis Unit (NPU) of the Institute for Animal Health). Experimental challenge of mice with tissue homogenates from CJD patients were carried out from 1995 and 2002 and did not directly involve the author of this thesis in either the planning or the animal husbandry aspects of this study. The period of PhD study began in October 2002. The author of this study has carried out all the laboratory tests on collected fixed and frozen tissue and performed all of the data analysis as described below unless otherwise stated.

2.1 Experimental mice

Experimental transmission of brain tissue obtained from patients diagnosed with CJD was carried out in mice from the colony maintained in the Category 3 containment facilities of the NPU/NPD. Three inbred mouse lines and one genetic cross were selected; these were RIII, C57BL, VM, and the F₁ cross between C57BL and VM mice (C57BL x VM). These mouse lines were selected because they are routinely included in TSE strain typing experiments at the NPU/NPD and have been well characterised after experimental challenge with many different TSE agents. These mouse lines differ in their prion protein genotype. RIII and C57BL mice are both of the *Prn-p^a* genotype; VM mice are of the *Prn-p^b* genotype and C57BL x VM mice are *Prn-p^{ab}*. The effect of the PrP gene on disease progression is described in full in Chapter 1, section 1.3.2. Mice within the Category 3 facility of the animal unit are all housed in individual ventilated cages (IVC). In this series of experiments, each cage held six mice. Strains and sexes of mice were kept separate and mice challenged with different TSE inocula were not mixed within cages. Individual mice were identified by an ear punch and a unique code for blind clinical assessment.

Information on each mouse was collated and stored in a purposely designed database (NPU/NPD e-mouse database).

2.2 Selection of CJD cases and tissues for transmission to mice

2.2.1 Human TSE case selection

All human tissues used in this series of transmission experiments were taken from the brain bank of the NCJDRSU. Forty cases of CJD (referred to in this thesis as CJD 1 – CJD 40) were selected for transmission. These comprised 27 sCJD and 13 vCJD cases. The patients were initially diagnosed on the basis of the clinical features of the disease and the diagnosis confirmed by neuropathological and Western blot examination of post-mortem CNS tissue. Inclusion criteria for cases were: the availability of frozen brain and peripheral tissues taken at post-mortem, appropriate consent and ethical approval for retention and research use (Lothian Research Ethics Committee (LREC) reference number LREC/2000/4/157), and a full range of clinical and genetic data on each CJD case. In addition, cases having a relatively short autopsy interval to minimise the effects of autolysis were preferred. Patient data for the forty CJD cases including age, gender, date of disease onset, disease duration, *PRNP* codon 129 genotype and PrP^{res} type are detailed in Table 2.1.

2.2.1.1 Brain tissue

Transmission experiments were carried out with brain tissue sampled from 27 sCJD patients with clinical onset ranging between 1980 and 1999. Therefore, this series of transmissions experiments included sCJD patients who had died both before and after the start of the BSE outbreak. Two of the sCJD cases (patients CJD 2 and CJD 4) were specifically selected as they had been dairy farmers from different UK farms who had potentially been exposed to BSE-infected cattle and BSE-contaminated animal feed because of BSE within their herds. The remaining 25 sCJD cases included all six possible combinations of *PRNP* codon 129 genotype and PrP^{res} type, as set out in the Parchi and Gambetti classification system for human TSEs (Parchi et al., 1999) and were selected as having clinical and pathological features that were typical for sCJD. In addition, transmission experiments were carried out with brain

Table 2.1: Patient data on clinical CJD cases selected for transmission to mice

*Case	Age at death	Gender	Disease Onset	Disease duration (months)	Diagnosis	PRNP codon 129 genotype	PrP ^{res} type (brain)
CJD 1	57	M	Sept 1990	1	sCJD	MM	1
CJD 2	61	M	Feb 1992	7	sCJD	MM	1
CJD 3	55	F	Sept 1992	1	sCJD	MM	1
CJD 4	64	M	Apr 1993	3	sCJD	MM	1
CJD 5	82	F	1983	4	sCJD	MM	1
CJD 6	57	F	1981	5.5	sCJD	MM	1
CJD 7	59	M	Jul 1995	3	sCJD	MM	1
CJD 8	78	M	1980	8	sCJD	MM	1
CJD 9	78	M	Dec 1995	4	sCJD	MM	1
CJD 10	86	F	Mar 1998	4	sCJD	MM	1
CJD 11	65	F	Dec 1997	9	sCJD	MM	1(+2A)
CJD 12	46	F	Dec 1996	17	sCJD	MM	2A
CJD 13	20	F	May 1993	54	sCJD	MV	1
CJD 14	78	M	Apr 1998	8	sCJD	MV	1
CJD 15	76	M	Jan 1999	5	sCJD	MV	1
CJD 16	54	M	Dec 1994	52	sCJD	MV	2A
CJD 17	66	F	Oct 1990	13	sCJD	MV	2A
CJD 18	64	F	Nov 1996	21	sCJD	MV	2A
CJD 19	54	M	Jul 1994	5	sCJD	VV	1
CJD 20	38	M	1984	15	sCJD	VV	1
CJD 21	28	M	Feb 1997	29	sCJD	VV	1
CJD 22	42	M	May 1995	10	sCJD	VV	1
CJD 23	53	M	1982	5	sCJD	VV	2A
CJD 24	69	M	1983	8	sCJD	VV	2A
CJD 25	77	M	1983	3.5	sCJD	VV	2A
CJD 26	53	M	Aug 1995	6	sCJD	VV	2A
CJD 27	47	M	Sept 1995	11	sCJD	VV	2A
CJD 28	29	F	Feb 1994	23	vCJD	MM	2B
CJD 29	30	F	Aug 1994	18	vCJD	MM	2B
CJD 30	31	M	Aug 1995	9	vCJD	MM	2B
CJD 31	35	F	Jul 1995	14	vCJD	MM	2B
CJD 32	41	F	July 1994	18	vCJD	MM	2B
CJD 33	25	M	Dec 1995	14	vCJD	MM	2B
CJD 34	36	M	Oct 1996	12	vCJD	MM	2B
CJD 35	23	F	Mar 1996	14	vCJD	MM	2B
CJD 36	33	F	Sept 1995	30	vCJD	MM	2B
CJD 37	36	F	Jul 1997	15	vCJD	MM	2B
CJD 38	39	M	May 1998	14	vCJD	MM	2B
CJD 39	26	M	Sept 2000	10	vCJD	MM	2B
CJD 40	30	F	Oct 2000	10	vCJD	MM	2B

* The identification reference (ID) for each case relates to this thesis only. Separate ID's were given to the same CJD cases analysed in previous publications. CJD 1 – CJD 6 and CJD 28 – CJD 30 corresponds to the CJD cases investigated in the original vCJD strain typing paper described by Bruce *et al* (2007). CJD 28 – CJD 37 correspond to the 10 vCJD cases investigated in Ritchie *et al* (2009).

tissue sampled from ten typical examples of vCJD from an early part of the vCJD epidemic (onsets between 1994 and 1997).

2.2.1.2 Peripheral lymphoid and neural tissues

In general, cases with a wide range of peripheral tissues available were selected for transmission. In the selection of vCJD lymphoid tissues (spleen, tonsil, lymph node and appendix), cases having the presence of PrP^{Sc} confirmed after immunohistochemical analysis were selected. In the sampling of tissues, trigeminal ganglia (TRG) were sampled from the left side. Dorsal root ganglia (DRG) were sampled from the lower thoracic/upper lumbar region. Bone for bone marrow extraction was sampled from the vertebral column. Peripheral nerves were sampled from the vagus nerve because of its potential involvement in disease pathogenesis.

2.2.1.3 Biological fluids

In addition to the experimental transmission of brain and peripheral tissues, mice were experimentally challenged with biological fluids and associated cells (buffy coat, plasma, and cerebrospinal fluid) taken from sCJD and vCJD patients. Buffy coat and plasma had been previously separated from whole blood samples by centrifugation. In the case of CSF, buffy coat and plasma, these samples were taken from patients during the clinical stage of disease and not at post-mortem. Differences in the time point at which these samples were taken may be important in the analysis of the results. Therefore, details of the time at which the biological fluids were taken, calculated as a percentage of the disease duration are detailed in Table 2.2.

2.2.2 Sampling of human TSE tissues

All human tissue sampling was carried out in the Class 1 microbiological safety cabinet in the Category 3* high-risk laboratory at the NCJDRSU by Professor James Ironside. For the experimental challenge of CJD brain tissue, a small piece of frozen grey matter (approximately 2 grams) from the frontal cortex or cerebellum of each CJD case was removed by aseptic technique, using sterilised single use disposable instruments. The size and weight of sample taken from peripheral tissues was dependent upon the availability of tissue for each case. Each sample was individually

labelled with the corresponding laboratory accession (RU) number assigned by the NCJDRSU to each CJD case and full and detailed records of the source and destination of the tissue taken. Tissues were transported to the NPU/NPD and stored at -80°C until required. Details of the individual tissues sampled from each CJD case for the inoculation in the mouse panel are shown in Table 2.3.

Table 2.2: Time points for the collection of biological fluids from CJD patients

Diagnosis	Case	Biological fluid	Time point extracted (% of the disease duration)
sCJD	CJD 10	Blood	76
	CJD 11	Blood	88
	CJD 15	Blood	69
	CJD 18	Blood	56
vCJD	CJD 31	Blood	87
	CJD 35	Blood	85
	CJD 36	Blood	81
	CJD 37	Blood	76
	CJD 38	Cerebrospinal fluid	43
	CJD 40	Cerebrospinal fluid	80

Table 2.3: Primary transmission of TSE infected tissues into panels of mice

	Case	Tissue type	Inoculation route	Inoculated mouse lines
sCJD	*CJD 1	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	*CJD 2	CB	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 3	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 4	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	*CJD 5	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 6	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 7	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 8	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 9	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 10	FC, BC, SPL	IC	RIII, VM
	CJD 11	FC, BC, PL	IC	RIII, VM
	CJD 12	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 13	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 14	CB	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 15	FC, BC	IC	RIII, VM
	CJD 16	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 17	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 18	FC, BC, PL	IC	RIII, VM
	CJD 19	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 20	FC	IC	RIII, C57BL, VM, C57BL x VM
	CJD 21	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 22	FC	IC/IP	C57BL
	CJD 23	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 24	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 25	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 26	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 27	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
vCJD	*CJD 28	CB	IC/IP	RIII, C57BL, VM, C57BL x VM
	*CJD 29	CB	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 30	CB	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 31	FC FC, BC, PL, SPL, TONS	IC/IP IC	RIII, C57BL, VM, C57BL x VM RIII, VM
	CJD 32	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 33	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 34	FC	IC/IP	RIII, C57BL, VM, C57BL x VM
	CJD 35	FC, BC, PL, TONS	IC	RIII, VM
	CJD 36	FC, BC, PL	IC	RIII, VM
	CJD 37	FC, BC, PL	IC	RIII, VM
	CJD 38	APP, BM, CSF, DRG, LN, PN, SC, TRG	IC	RIII
	CJD 39	APP, BM, DRG, LN, PN, SC, TRG	IC	RIII
	CJD 40	APP, BM, CSF, DRG, LN, PN, SC, TRG	IC	RIII

APP – appendix, BC – buffy coat, BM – bone marrow, CB - cerebellar cortex, CSF – cerebrospinal fluid, DRG – dorsal root ganglia, FC – frontal cortex, LN – lymph node, PL – plasma, PN – peripheral nerve, SC – spinal cord, SPL – spleen, TONS – tonsil, TRG – trigeminal ganglia

* subsequent mouse-to-mouse passages of brain isolates was carried out from these cases

2.3 Preparation of inocula and inoculations

All inoculations were carried out at the NPU/NPD under an appropriate Home Office project licence (PPL 60/3132 and PPL 60/3380). Inoculum preparation and injections were carried out in a Class II safety cabinet within the category 3 containment facility. Injections were carried out by Professor Moira Bruce, Mrs Irene McConnell, Mrs Val Thomson and Miss Dawn Drummond.

2.3.1 Primary transmission

In the inoculation of tissue or biological fluids from CJD patients into mice, a 'species barrier' exists between the donor and recipient species. Previous transmission experiments suggest that the attack rate (number of mice that develop clinical signs within a defined period) in such primary transmission is often low with extended incubation periods. In order to maximise the chance of transmission in this series of experiments:

- Wherever possible, a large number of mice (20-24 mice of each strain) were inoculated with each CJD inoculum to compensate for losses due to intercurrent death during an extended incubation period.
- Inoculation by a combination of intracerebral (IC) and intraperitoneal (IP) routes was carried out in 29 of the 38 primary transmissions from CJD brain homogenates.
- Inoculations were carried out with a relatively concentrated 10% weight/volume (w/v) tissue homogenate.

Inocula were prepared using the standard methods employed at the NPU/NPD (Dickinson et al., 1968; Bruce et al., 2004). Tissue samples were thawed, re-weighed and transferred to a sterile tissue grinder. Tissues were homogenised in a 0.9% sterile physiological saline solution (TPS medical, UK; product SC005) to give a 10% wet weight concentration and transferred into sterilised, sealable glass containers and stored at -20°C until required. Due to the extreme resistance of the TSE agent to conventional decontamination methods, all glassware was disposed of after single use. Cerebrospinal fluid, buffy coat and plasma, were inoculated into mice undiluted.

Bone marrow was prepared by flushing the bone through with 1ml physiological saline. Therefore the dilution of the bone marrow inoculum was unknown. Prior to injection, homogenates were thawed and re-suspended by being drawn repeatedly through a series of graded needles. Mice were inoculated via a single IC injection or by a combination of IC and IP routes using procedures described below.

2.3.2 Intracerebral inoculation of mice

Mice were injected intracerebrally with 20µl volume of inoculum under halothane anaesthesia (fluothane). Injections were made to the right of the midline of the skull through the parietal bone. A 26G needle was used for the injections with a needle guard attached preventing the needle penetrating more than 2mm into the brain.

2.3.3 Intraperitoneal inoculation of mice

Mice were injected via the IP route with 100µl volume of inoculum while under halothane anaesthesia. Injections were made into the ventral abdominal cavity using a 26G needle.

The mouse lines inoculated with each CJD brain homogenate and the routes of inoculation are detailed in Table 2.3. In the majority of experiments, control animals were included, with six mice of each mouse line injected with 0.9% sterile physiological saline via the same inoculation route. After inoculation, the general health of the mice was monitored daily. All remaining unused inoculum and residual frozen tissue was returned to the NCJDRSU and stored at –80°C for biochemical analysis.

2.3.4 Secondary and serial passages in mice

Secondary mouse-to-mouse passage of isolates derived from brain samples of three sCJD cases (CJD 1, CJD 2 and CJD 5) and two vCJD cases (CJD 28 and CJD 29) were carried out in RIII, C57BL and VM mice. All three sCJD cases selected for sub-passage were of the MM1 subtype, the most common subtype of sCJD, accounting for approximately 70% of all sCJD cases (Alperovitch et al., 1999).

For secondary mouse passage, inoculum was prepared as previously described for primary transmissions (chapter 2, section 2.3.1), from the brain of a single RIII, C57BL or VM mouse which, where possible, had received a positive score for both clinical and neuropathological signs of TSE disease following primary transmission. In the absence of mice with a positive score for clinical disease, mice that had received a positive score for TSE-specific vacuolar pathology were selected. With the removal of the 'species barrier' on secondary passage (Dickinson, 1976; Bruce et al., 1994) and the expectation that transmission would now be more efficient, inoculum was prepared at a lower concentration of 1% (w/v) in physiological saline and injected via a single IC injection. For each inoculum, smaller groups of 10-12 mice were inoculated. A further serial mouse-to-mouse passage was carried out from each secondary passage using the same protocol.

2.4 Clinical monitoring of mice experimentally challenged with TSEs

In all primary transmissions, formal clinical scoring of mice for signs of clinical TSE disease was carried out weekly from 250 days post injection (dpi) until the end of the natural life span of the mice, by suitably qualified NPU/NPD animal facility staff. This time-point is approximately 50 days before the clinical end-point of the shortest incubation periods observed in non-transgenic mice after primary challenge with a TSE. Clinical scoring for sub-passage experiments was started earlier, at 50dpi because incubation periods generally shorten with the removal of the 'species barrier'.

Clinical signs of TSE disease in mice are variable and often begin with subtle changes in behaviour prior to definite neurological disease. Some of the clinical signs

included lethargy, hyperactivity, changes in gait, aggression, weight loss or gain and scratching of the skin. Each week inoculated mice were given a score of “unaffected”, “possibly affected” or “definitely affected”. Mice were sacrificed at either clinical end-point for TSE disease and allocated a positive clinical score; or the full life span of the mice if they do not develop clinical TSE disease and allocated a negative clinical score. In some experiments, a termination date was set in which all remaining animals in the experiment were culled. Clinical end-point was determined in the following ways:

- The day on which the mouse received two consecutive “definitely affected” scores.
- The day on which the mouse received a second “definitely affected” rating in three consecutive weeks.
- The animal had been found dead in the cage having received a “definitely affected” score at the previous week’s scoring.

2.4.1 Incubation period

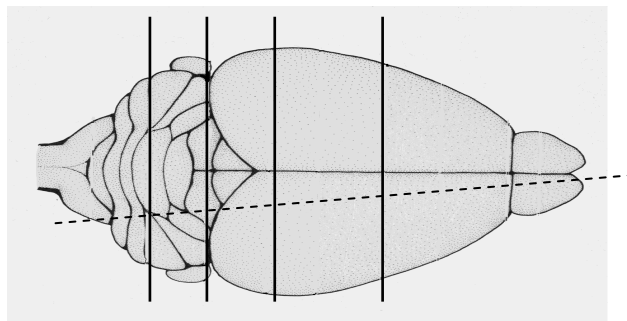
Incubation period in mice challenged with individual TSE isolates is a major transmission characteristic that helps define agent strain (Dickinson and Meikle, 1971; Bruce et al., 1991). Incubation periods were calculated as the period in days between initial infection (date of injection) and the date at which mice were sacrificed at clinical end-point of TSE disease (date of death). Mean incubation periods were then calculated for individual groups within an experiment. Survival times were calculated for mice sacrificed due to intercurrent disease or at the end of their natural life span where no clinical signs were observed. In primary transmissions, mice sacrificed as a result of intercurrent disease before 250dpi, the start of formal clinical scoring, were excluded from the analyses. This time-point was shortened to 50dpi for the further mouse-to-mouse passages due to the removal of the ‘species barrier’.

2.5 Harvesting of mouse CNS tissues at post-mortem

At post-mortem, brains were removed aseptically and assigned a unique brain pathology (BP) number for identification purposes and for blind histological analysis. For the majority of mice, brains taken at post-mortem were immersed in 10% formal saline for histological analysis. However in a proportion of mice a lateral third of the brain was sliced and frozen and stored at -80°C for either biochemical analysis of PrP^{res} type or for further passage in mice as shown in Figure 2.1. The remaining two thirds of the brain were immersed in 10% formal saline for histological analysis.

Figure 2.1: Trimming levels for fixed mouse brain.

The dashed line shows the lateral portion of the brain that is routinely sliced away and frozen for biochemical analysis of PrP^{res} or for further passage in mice. Solid lines show the trimming levels providing four section levels for histological analysis (Image adapted from Bruce et al., 2004).



2.6 Histological Analysis

Neuropathological examination of CNS tissue remains the definitive method for the diagnosis TSEs. In experimental mouse models, analysis of spongiform vacuolation and PrP deposition within the mouse brain not only confirms the successful transmission of a TSE, but also plays an important role in defining agent strain.

2.6.1 Fixation, embedding and sectioning of mouse brain for histological techniques

Mouse brains for histological analysis were fixed in 10% formal saline for at least 48 hours, before immersing in 98% formic acid for 1 hour, a step introduced to reduce the titre of the infectious agent (Brown et al., 1990). Tissues were then re-immersed in 10% formal saline for a further 48 hours. Brains were trimmed coronally at four standard rostro-caudal levels to give five brain slices (Figure 2.1). These levels are precise in order to expose the nine grey matter regions and three white matter regions required for lesion profiling (see section 2.6.3). Tissues were then processed into paraffin wax and embedded exposing the cut surface of the tissue. Serial sections (approximately 10) were cut at a thickness of 5µm. Sections were placed onto electrostatically charged microscope slides (VWR international, UK; product 631-0108) and incubated at 55°C for at least 24 hours.

2.6.2 Haematoxylin and eosin staining

To confirm the presence of TSE-specific vacuolation and undertake lesion profiling, haematoxylin and eosin (H&E) staining was performed on a single formalin fixed paraffin brain section of all inoculated mice as follows:

Paraffin embedded brain sections were de-paraffinised in xylene for 5 minutes followed by stepwise re-hydration (5 minute incubations) in absolute alcohol, 74% industrial methylated spirits (IMS) and 70% IMS. Sections were immersed in haematoxylin solution (Surgipath Europe Ltd, UK; product 01542E) for 3 minutes before a thorough wash in water. After a single dip in a 1% acid alcohol solution [99mls 70% alcohol; 1ml concentrated HCL], sections were dipped three times in lithium carbonate solution [300ml tap water; 5-10ml saturated aqueous lithium carbonate (Fisher Scientific, UK; product L/2100/50)] and stained in eosin solution (Surgipath Europe Ltd, UK; product 01600E) for 3 minutes. Following a final rinse in water, sections were de-hydrated by immersing (approximately 10 seconds) in 70% IMS, 74% IMS and absolute alcohol. Sections were cleared in xylene, cover slipped with pertex mountant (Leica Microsystems, UK; product 08707E) and left to harden overnight. In order to analyse the slides outside the laboratory, sections were immersed in 96% formic acid for 5 minutes and washed well in water.

2.6.3 Assessment of TSE-specific vacuolation – The lesion profile

Along with incubation period, the targeting and severity of TSE-specific vacuolation provide a “signature” for the biological properties of the agent. Lesion profiling is a semi-quantitative assessment of the severity of TSE-specific vacuolar pathology in nine specified grey matter regions and three white matter regions of the mouse brain (Fraser and Dickinson, 1968) (Figure 2.2). In each of the nine grey matter scoring regions, the severity of vacuolation is scored on a scale of 0-5. The definition of vacuolation scores in the grey matter is shown in Table 2.4. Vacuolation scores from a minimum of five mice in a single experimental group were required in order to construct a reliable lesion profile. All mice in this transmission series, regardless of clinical status were scored for the presence of TSE-specific vacuolation. To ensure consistency of results all scoring was carried out by Aileen Boyle at the NPU/NPD.

Figure 2.2: Lesion profiling scoring regions.

The nine grey matter scoring regions (numbers 1-9) and three white matter scoring region (WM1-WM3) are indicated. Grey matter scoring regions are: (1) dorsal medulla, (2) cerebellar cortex of folia adjacent to fourth ventricle, (3) superior colliculus, (4) hypothalamus, (5) medial thalamus, (6) hippocampus, (7) septum, (8) and (9) medial areas of the cerebral cortex at levels shown. White matter areas are: (1) inferior and middle cerebral peduncles, (2) decussation of superior cerebellar peduncles, (3) cerebral peduncles. The dashed lines show the lateral portion of the brain sliced and snap frozen (Image adapted from Bruce et al., 2004).

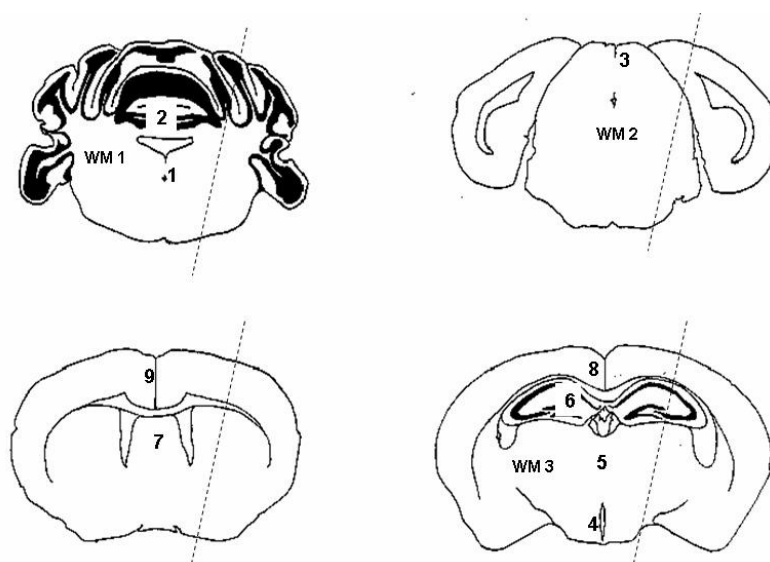


Table 2.4: Definition of vacuolation scores
(From Bruce et al., 2004)

Vacuolation score	Description
0	No vacuoles
1	A few vacuoles widely and unevenly scattered, not convincingly TSE specific pathology
2	A few vacuoles evenly scattered, more than for score 1 and convincingly diagnostic for TSE
3	Moderate numbers of vacuoles, evenly scattered
4	Many vacuoles with some confluence
5	Dense vacuolation with most of microscopic field confluent, lace like appearance

2.6.4 Immunohistochemical detection of PrP in CNS tissues from mice experimentally challenged with TSE

Detection of PrP in formalin fixed tissue sections was carried out using the anti-PrP antibody 6H4 (Prionics, Switzerland; product 01-010) in combination with the highly sensitive catalyzed signal amplification (CSA) (DAKO, UK; product K1500) system which is used at the NCJDRSU in the detection of PrP in human tissue sections. In each immunohistochemical run, a paraffin embedded section from a VM mouse experimentally challenged with the 87V strain of scrapie was included as a positive control for the antibody.

Paraffin embedded tissue sections were de-paraffinised in xylene for 5 minutes followed by stepwise re-hydration in absolute alcohol, 74% IMS and 70% IMS (5 minute incubations). Endogenous peroxidase activity was blocked by immersing sections in 1% hydrogen peroxidise (Fisher Scientific, UK; product BP2633-500) in methanol (Fisher Scientific, UK; product M/3950/17) for 30 minutes. In order to retrieve PrP antigen binding sites from the formalin fixed tissues, sections were autoclaved in distilled water (dH₂O) at 121°C for 10 minutes followed by immersion in 96% formic acid (Fisher Scientific, UK; product F/1850/PB17) for 5 minutes. Sections were washed in Tris-buffered saline (TBS) [50mM Tris; 150mM NaCl; pH7.6] before blocking with normal rabbit serum (NRS) (Vector laboratories, UK; product S-5000) diluted 1:5 in TBS for 20 minutes. Blocking of any non-specific binding of avidin-biotin was carried out with an avidin-biotin blocking kit (Vector laboratories, UK; product SP-2001). Sections were incubated for 15 minutes in the avidin solution followed by a 15 minute incubation in biotin solution. Sections were then incubated in the primary antibody 6H4, diluted 1:8000 in TBS for 1 hour. After a wash in TBS, immunolabelling was completed using the CSA kit with 15-minute incubations in biotinylated link, streptavidin biotin complex reagent [Prepare at least 30 minutes prior to use: 1 drop streptavidin-biotin complex reagent A; 1 drop of streptavidin-biotin reagent B; 1 ml of streptavidin-biotin complex diluent], amplification reagent and streptavidin-HRP. Washes in TBS were carried out between each reagent. After a final wash, staining was visualised with 3, 3' diaminobenzidine (DAB). The DAB was prepared immediately before use from the

CSA kit [10 drops of substrate buffer concentrate; 10 ml of dH₂O; 1 DAB chromagen tablet. The DAB was activated immediately before use by adding 1 drop of substrate hydrogen peroxide per 2 ml of DAB solution (DAKO, UK: product K1500)]. Sections were washed well in water before lightly counterstained (immerse in haematoxylin solution for 20 seconds, wash in water, dip once in a 1% acid alcohol solution, rinse in water and dip 4 times in saturated aqueous lithium carbonate solution). After a wash in water sections were dehydrated by immersing (approximately 10 seconds) in 70% IMS, 74% IMS and absolute alcohol. After clearing in xylene, sections were cover slipped with pertex and left to harden overnight. In order to analyse the slides outside the laboratory, sections were immersed in 96% formic acid for 5 minutes and washed well in water.

2.6.5 Immunohistochemical detection of astrocytes in CNS tissue from mice experimentally challenged with TSE

Astrocytic proliferation, a common pathological feature of TSEs, is marked by increased glial fibrillary acidic protein (GFAP) immunostaining. Mice were immunolabelled with the anti-GFAP antibody, GFAP (Dako, UK; product Z0334) in combination with a Vectastain rabbit elite ABC kit (Vector laboratories, UK; product PK-6101) in order to visualise astrocytosis within the mouse brain after experimental challenge with the different TSE inocula. Immunolabelling was carried out on vCJD challenged mice as follows:

Paraffin embedded tissue sections were de-paraffinised in xylene for 5 minutes followed by stepwise re-hydration in absolute alcohol, 74% IMS and 70% IMS (5 minute incubations). Endogenous peroxidase activity was blocked by immersing sections in 1% hydrogen peroxidise in methanol for 30 minutes. Sections were washed in TBS before blocking in normal goat serum (NGS) [1 drop of NGS in 10mls TBS]. Excess NGS was drained off and sections incubated in the primary antibody GFAP diluted 1:800 in TBS for 45 minutes at room temperature. After a wash in TBS, sections were incubated in anti-rabbit biotinylated secondary for 30 minutes [1 drop of biotinylated anti-rabbit to 10mls of NGS] before a further wash in TBS. Immunolabelling was completed by incubating sections in ABC elite reagent for 30 minutes [prepared at least 30 minutes prior to: add 5mls TBS/BSA; 1 drop reagent A; 1 drop reagent B]. Staining was then visualised with DAB and sections counterstained and cover slipped for analysis as described in the CSA detection method of PrP in section 2.6.4.

2.6.6 Microscopy and image capture.

Staining of paraffin embedded tissue sections was analysed with a Leica DMR microscope and image capture carried out with a Leica DFC 500 camera in combination with the Leica LAS image acquisition software (Leica Microsystems, UK).

2.7 Biochemical typing of PrP^{res} in frozen tissues

PrP^{res} typing of brain inocula and frozen brain tissue was carried out using the standard diagnostic Western blot protocol carried out at the NCJDRSU for the detection and classification of PrP^{res} in human CNS tissue (Head et al., 2004a). As discussed in Chapter 1, section 1.10.1.2, investigations of PrP^{res} types in sCJD described in this thesis follow the Parchi and Gambetti classification system for human TSEs. In this classification system, two PrP^{res} types are associated with sCJD differing in the migration of the unglycosylated fragment of the protein; type 1 where the unglycosylated PrP^{res} has a molecular weight of around 21 kDa and type 2, where the unglycosylated PrP^{res} fragment is slightly smaller with a mobility of around 19kDa (Parchi et al. 1996). The migration pattern of the unglycosylated fragment of the protein in sCJD type 2 cases is indistinguishable to that of vCJD. However, vCJD is distinguished from sCJD type 2 cases by the predominance in the diglycosylated form of the protein. To indicate this difference in glycoform ratio the type 2 PrP^{res} associated with sCJD is termed type 2A and the type 2 PrP^{res} associated with vCJD is termed type 2B. Therefore in all Western blot runs performed in this thesis analysing both human and murine samples, diagnostic reference standards from a sCJD MM1 patient and a vCJD (MM2B) patient were included with every Western blot run serving as an appropriate reference control for both fragment mobility and glycoform ratio.

2.7.1 Sample preparation for Western blot analysis

Preparation of tissue for Western blot analysis was carried out in the Class 1 Microbiological Safety Cabinet within the High Risk Laboratory of the NCJDRSU as follows:

2.7.1.1 Preparation of PrP^{res} from tissue inocula

Residual inocula from the transmission series were vortexed and a 0.45ml sample removed and supplemented with 0.05ml of 10X extraction buffer [200mM Tris pH7.4; 5% Nonident P40; 5% Sodium deoxycholate]. After a brief vortex, insoluble material was pelleted by spinning samples at 2000rpm for 5 minutes at 4°C using a microcentrifuge (Eppendorf AG, UK; product 5417R) with a fixed angle motor

(Eppendorf AG, UK; product F-45-30-11). The supernatant was then aspirated using an aerosol resistant tip into a fresh eppendorf tube and the remaining pellet frozen at -20°C. A 0.2ml volume of the supernatant was then aspirated into a fresh eppendorf and proteinase K (PK) (VWR, UK; product 395092L) added to a final concentration of 50µg/ml. PK treated samples were then incubated at 37°C for 1 hour before stopping the reaction with the addition of Pefabloc™ (Roche, UK; product 11429868001) to a final concentration of 1mM. The PK treated sample along with any residual supernatant were frozen at -20°C until required.

2.7.1.2 Preparation of PrP^{res} from frozen brain tissue

A small piece (approximately 100mg) of frozen grey matter enriched brain tissue (human or mouse) was sampled and placed in a sterile eppendorf. The precise weight of each brain sample was calculated and sufficient 1X extraction buffer [20mM Tris pH7.4; 0.5% Nonident P-40; 0.5% Sodium deoxycholate] added to give a 10% (wt/vol) brain homogenate. Tissues were then homogenised in the 1X extraction buffer using a disposable micropestle. Insoluble material was pelleted by spinning at 2000rpm for 5 minutes at 4°C. The supernatant was aspirated using an aerosol resistant tip into a fresh eppendorf tube and the remaining pellet frozen at -20°C. A 0.2ml volume of the supernatant was then removed and PK treated as described in the preparation of PrP^{res} from tissue inocula (section 2.7.1.1). After stopping the reaction with Pefabloc™, the PK treated sample was frozen at -20°C until required.

2.7.1.3 Centrifugal concentration of PrP^{res}

Centrifugal concentration is an optional step used for increased sensitivity in the Western blot detection of PrP^{res}. After PK treatment, 100µl of the PK treated sample was given a brief vortex before centrifugation at 14,000rpm for 1 hour at 4°C. The supernatant was removed and the pellet re-suspended in 5µl of 4X NuPAGE LDS sample buffer (Invitrogen, UK; product NP007) supplemented with 15µl of 1X extraction buffer (20mM Tris pH7.4; 0.5%NP-40; 0.5% Sodium deoxycholate). After a short vortex, the sample was boiled at 100°C for 10 minutes and stored at -20°C until required.

2.7.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

PK treated samples were thawed and given a short vortex before removing a 5µl volume and adding to 5µl of 4X NuPAGE LDS sample buffer, supplemented with 10µl of 1X Extraction buffer. After a brief vortex, samples were denatured by boiling at 100°C for 10 minutes before a brief spin at 14,000rpm. In addition to the human diagnostic reference standards, protein molecular weight markers were included in each run [1µl Magic MarkerTM XP (Invitrogen, UK; product LC5602); 5µl Benchmark (Invitrogen, UK; product 10748-010); 9µl of 1X Extraction buffer; 5µl of NuPAGE LDS sample buffer]. Molecular weight markers were not boiled. Proteins were then separated by running at 200V (constant current) for 55 minutes in 1X NuPAGE MES running buffer [50mls 20X running buffer (Invitrogen, UK; product NP0002-02); 950mls dH₂O] within a Western blot running tank.

2.7.3 Western blot transfer

Proteins separated by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-rad, UK; product 162-0177) at 30V (constant current) for 1 hour using the NuPAGE transfer system. Transfers were carried out in 1X NuPAGE transfer buffer [25ml 20X NuPAGE transfer buffer (Invitrogen, UK; product NP0006-1); 375ml dH₂O, 100ml methanol].

2.7.4 Detection of PrP bound to PDVF membrane

Membranes were rinsed in TBST [100mls 10X TBS (Sigma-Aldrich, UK; product T5912); 900mls dH₂O; 0.1% Tween 20 (Sigma-Aldrich, UK; product P9416)] before incubating in 5% powdered milk/TBST for 45 minutes at room temperature or overnight at 4°C. Membranes were then washed (3 x 5 minutes) in TBST prior to incubating in the primary anti-PrP antibody 6H4 diluted 1:25 000 in TBST for 1 hour at room temperature. After a further wash, immunolabelling was completed by incubating the membrane in the anti-mouse IgG linked horseradish peroxidase-conjugated secondary antibody (GE Healthcare, UK; product NA9310) diluted 1:40,000 in TBST for 1 hour at room temperature. Immunodetection was carried out

by incubating the membrane with the chemiluminescent substrate ECL plus [50µl of reagent B per 2 mls of reagent A (GE Healthcare, UK; product RPN2132)]. After draining off the excess ECL plus, membranes were exposed to Hyperfilm ECL (GE Healthcare, UK; product 28-9068-37) for 30 seconds, 3 minutes and 30 minutes before the films were developed in an automatic film hyperprocessor (Konica Minolta SRX-101A medical film processor).

2.7.5 Densitometry and PrP^{res} typing

Once developed, X-ray films were scanned using a GS-700 or 800 Densitometer (Biorad, UK; product 170-7980) and densitometry performed using the Biorad Quantity One Analysis software (version 4.4.1). The glycoform ratio and mobility of the unglycosylated band of samples under investigation were directly compared to the diagnostic reference standards (sCJD MM1 and vCJD MM2B).

Chapter 3: Characterisation of the inocula used in wild-type mouse transmission study

3.1 Objective

1. To confirm the presence of PrP^{res} within inocula used in the experimental transmission of CJD to wild-type mice.
2. To confirm the original brain PrP^{res} isotyping result using the actual inocula used for transmission studies.

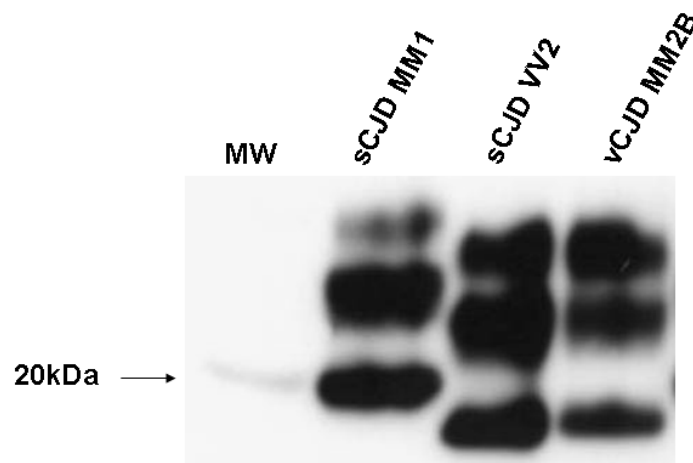
3.2 Introduction

The existence of individual TSE strains raises the question of how a single misfolded protein can encode all of the information required for the demonstrable phenotypic diversity of TSEs (Farquhar et al., 1998). The ‘protein only’ hypothesis proposes that strain-specific biological properties are enciphered in the different conformations and glycosylation states of PrP^{Sc} (Collinge et al., 1996; Telling et al., 1996). These different conformers and glycotypes are usually distinguished by the analysis of the proteinase resistant core of PrP^{Sc} (referred to as PrP^{res}) after limited digestion with PK and analysis by Western blotting in an approach commonly referred to as ‘molecular strain typing’ (Collinge et al., 1996; Parchi et al., 1996; Parchi et al., 1999). In the classification system of human TSEs described by Parchi and Gambetti, two major PrP^{res} types have been reported among cases of sCJD: type 1 where the unglycosylated PrP^{res} has a molecular weight of around 21 kDa and type 2A, where the unglycosylated PrP^{res} is slightly smaller with a mobility of around 19kDa (Parchi et al. 1996). A representative Western blot showing the fragment mobility and glycoform ratio of PrP^{res} from a diagnostic reference standard from a sCJD MM1 patient and a sCJD VV2 patient is shown in Figure 3.1 These two biochemically distinct PrP^{res} types have been associated with different sCJD phenotypes (Parchi et al. 1999). In order for these biochemical types to be considered as being properties of separate strains of the agent causing sCJD, the biochemical properties must be maintained on serial passage in mice.

Recently the significance of biochemical typing in CJD has been questioned with the observation of type 1 and type 2A co-occurring within the same brain in up to a third of sCJD patients examined (Puoti et al., 1999; Schoch et al., 2006; Uro-Coste et al., 2008). These studies examined multiple small samples from different regions of the brain from individual sCJD patients, and it has been suggested that regional variation of PrP^{res} types may be the rule rather than the exception in CJD. This has been further supported with reports of regional variation in PrP^{res} types within cases of iCJD (Head et al., 2004b; Uro-Coste et al., 2008) and fCJD (Haik et al., 2004). In contrast to sCJD, only a single PrP^{res} type (type 2B) has been detected within the brain of vCJD patients, defined by a characteristic predominance of the diglycosylated form of PrP^{res} and an apparent molecular weight of around 19kDa for the unglycosylated form of PrP^{res} (Figure 3.1) (Collinge et al. 1996; Head et al 2004a). A similar glycosylation pattern has been found in cases of natural BSE and other BSE related conditions (Collinge et al., 1996). This BSE “glycoform signature” supports the hypothesis that vCJD and BSE are linked by a common agent. In recent studies using PrP^{res} “type 1 selective” antibodies some researchers have claimed to detect low levels of type 1 PrP^{res} in vCJD and BSE brain (Yull et al., 2006), but the interpretation of these results remains controversial on technical grounds (Notari et al., 2007).

Figure 3.1: PrP^{res} in sCJD and vCJD diagnostic reference standards

Western blot analysis of PrP^{res} in frontal cortex samples of a sCJD MM type 1, a sCJD VV type 2 and a vCJD (MM) type 2B diagnostic reference standard. Tissues are analysed after digestion with proteinase K and blots probed with the anti-PrP antibody 6H4. Molecular weight markers (MW) are shown in kDa.



If strain-specific properties are enciphered in the different conformations and glycosylation states of PrP as outlined in the 'protein only' hypothesis, establishing the actual PrP^{res} type of inocula used in formal strain typing experiments is of fundamental importance to the interpretation of the results. In the series of experiments described in this study, CJD cases for transmission were selected according to the PrP^{res} type identified in brain tissue taken at post-mortem. However, in the preparation of inocula for transmission, the brain from each CJD case was re-sampled, raising the possibility that the PrP^{res} type of the specimen for transmission may be different from the specimen used for diagnosis as a result of regional variation in the brain. In order to accurately interpret results from this study, it was therefore necessary to carry out Western blotting on inocula from each sCJD source as a means of establishing the actual PrP^{res} type in the homogenate used to inoculate the mice. Although only a single PrP^{res} type has been reported in the brain of vCJD patients, when analysed by conventional Western blotting, inocula from the vCJD transmissions were also analysed by Western blotting.

In addition to the experimental transmission of brain tissue, this study investigated attempted transmissions from a wide range of lymphoid tissues, peripheral neural tissues and biological fluids, to establish whether TSE infectivity can be detected in tissues other than brain in both sCJD and vCJD. Accumulation of PrP^{Sc} has been described in a wide range of lymphoid and peripheral neural tissues in vCJD and to a much lesser extent in certain sCJD cases (Glatzel et al., 2003; Haik et al., 2003; Head et al., 2004b; Peden et al., 2006; Peden et al., 2007; Notari et al., 2010). However, studies showing the correlation between PrP^{Sc} accumulation and infectivity are limited. Infection of spleen and tonsil tissue in vCJD patients has been previously confirmed by bioassay in RIII mice and has indicated that infectivity levels are a hundred to a thousand times lower than those demonstrated in brain (Bruce et al., 2001). Furthermore, immunohistochemistry and Western blot studies have shown that the distribution of PrP^{Sc} within peripheral tissues of vCJD is much more heterogeneous than those found in the brain (Joiner et al., 2002; Hilton et al., 2004a; Head et al., 2004b). Immunohistochemical analysis of PrP^{Sc} in lymphoid tissues from vCJD patients at the end-stage of disease showed positively stained germinal centres

in a patchy distribution (Head et al., 2004b). This finding mirrors the irregular presence of PrP^{Sc} in the appendix in vCJD patients as detected by Western blot analysis (Joiner et al., 2002), with positivity detected in one out of two specimens at levels around 0.5% of that in the brain. These findings suggest that there may be sampling issues associated with the transmission of CJD infected peripheral tissues, in that PrP^{Sc} (and infectivity) may be present in the tissues but that the small samples taken for inoculation might not include areas where foci are most frequent. Consequently, even in those CJD tissues which were confirmed as having PrP by immunohistochemistry, establishing the presence of PrP^{res} within inocula prepared from these tissues is crucial to the interpretation of results. Therefore Western blotting was carried out on residual inocula as a means of establishing whether PrP^{res} was present in the tissue homogenates inoculated into the mice.

3.3 Experimental results

3.3.1 Confirmation of PrP^{res} type of human brain tissue homogenates inoculated into mice

In this investigation of agent strain in CJD, 38 primary transmission experiments were carried out from brain homogenates prepared from 37 patients diagnosed with CJD (27 sCJD patients and 10 vCJD patients). Three inbred mouse lines (RIII, C57BL and VM) and one cross C57BL X VM were challenged with individual brain homogenates as described in Chapter 2, section 2.3. Not all inocula were available for analysis; so Western blot analysis was carried out using the following order of preference;

- 1. Human brain inoculum.** Homogenate used in the inoculation of mice was the preferred and was considered to be the definitive method of determining the PrP^{res} type of the brain homogenate.
- 2. Residual frozen brain tissue used for the preparation of inoculum.** In the absence of inoculum, Western blotting was carried out on a sample prepared from any unused frozen brain tissue sent to the NPU/NPD for transmission.
- 3. CJD brain bank.** In the absence of any residual inoculum or frozen brain sample, the original brain held at the NCJDRSU was re-sampled. The sample for Western blot was taken from the same brain region as that previously sampled for transmission.

Inoculum was available from 30 of the 38 primary transmissions for PrP^{res} isotyping. Residual tissue from the brain sample sent to NPU/NPD was analysed for four experiments with the original brain held at the NCJDRSU brain bank re-sampled for the remaining four experiments. The tissue type analysed from each CJD case and the resulting PrP^{res} type after Western blotting is detailed in Table 3.1.

PrP^{res} was readily detected in all brain extracts analysed. A similar intensity in the PrP^{res} banding pattern was observed between the different brain extracts analysed. Thirty-seven of the 38 brain extracts analysed confirmed the PrP^{res} type determined at diagnosis (Table 3.1). Crucially for this study, these results confirmed the transmission of brain tissue from all six possible combinations of *PRNP* codon 129 genotype and PrP^{res} type used in the classification of sCJD (Parchi et al., 2009). The number of individual sCJD cases of each *PRNP* codon 129 genotype/PrP^{res} type combination used in a primary transmission is summarised in Table 3.2. A representative Western blot from the analysis of inoculum from each of these six possible CJD subgroups is shown in Figure 3.2.

The single contradictory result from the Western blot analysis was from a sCJD patient (CJD 11) who was originally characterised as having PrP^{res} type 1 in the extract of frontal cortex tissue tested during diagnosis, but who was subsequently found to have both PrP^{res} type 1 and 2A in the brain when a large cortical specimen was taken to prepare sCJD reference standards by the National Institute of Biological Standards and Control (Minor et al., 2004). Neuropathological examination of post-mortem frontal cortex tissue from this case, the brain region used in the preparation of inoculum, showed pathological changes consistent with those found in both sCJD MM1 and MM2 cases (Figure 3.3). This case has since been re-classified as sCJD MM1 + 2 as a result of the subsequent Western blot findings. In this study, re-analysis of the original diagnostic specimen homogenate showed that although it is predominantly type 1, it also contains some type 2 PrP^{res} (Figure 3.4). Western blot analysis of the actual inoculum from this case detected PrP^{res} solely with an electrophoretic mobility and glycosylation pattern of sCJD type 2A (Figure 3.4).

Table 3.1: Confirmation of PrP^{res} in brain homogenates inoculated into mice

	Case	Codon 129 genotype	Reported PrP ^{res} type in diagnostic specimen	Confirmed PrP ^{res} type in inoculum	Tissue type tested
sCJD	†CJD 1	MM	1	1	human brain inoculum
	†CJD 2	MM	1	1	human brain inoculum
	CJD 3	MM	1	1	human brain inoculum
	CJD 4	MM	1	1	human brain inoculum
	†CJD 5	MM	1	1	human brain inoculum
	CJD 6	MM	1	1	human brain inoculum
	CJD 7	MM	1	1	human brain inoculum
	CJD 8	MM	1	1	CJD brain bank
	CJD 9	MM	1	1	human brain inoculum
	CJD 10	MM	1	1	residual brain sample
	CJD 11	MM	1(+2A)	2A	human brain inoculum
	CJD 12	MM	2A	2A	CJD brain bank
	CJD 13	MV	1	1	human brain inoculum
	CJD 14	MV	1	1	human brain inoculum
	CJD 15	MV	1	1	human brain inoculum
	CJD 16	MV	2A	2A	human brain inoculum
	CJD 17	MV	2A	2A	human brain inoculum
	CJD 18	MV	2A	2A	residual brain sample
	CJD 19	VV	1	1	human brain inoculum
	CJD 20	VV	1	1	CJD brain bank
	CJD 21	VV	1	1	human brain inoculum
	CJD 22	VV	1	1	human brain inoculum
	CJD 23	VV	2A	2A	human brain inoculum
	CJD 24	VV	2A	2A	human brain inoculum
	CJD 25	VV	2A	2A	CJD brain bank
	CJD 26	VV	2A	2A	human brain inoculum
	CJD 27	VV	2A	2A	human brain inoculum
vCJD	†CJD 28	MM	2B	2B	residual brain sample
	†CJD 29	MM	2B	2B	human brain inoculum
	CJD 30	MM	2B	2B	human brain inoculum
	*CJD 31	MM	2B	2B	human brain inoculum
	CJD 32	MM	2B	2B	human brain inoculum
	CJD 33	MM	2B	2B	human brain inoculum
	CJD 34	MM	2B	2B	human brain inoculum
	CJD 35	MM	2B	2B	human brain inoculum
	CJD 36	MM	2B	2B	human brain inoculum
	CJD 37	MM	2B	2B	residual brain sample

* Two separate transmission experiments were carried out from this CJD case.

† Further serial mouse-to-mouse passages carried out from the primary transmissions.

Table 3.2: CJD cases used in the brain tissue transmission series

PrP^{res} type	<i>PRNP</i> codon 129 genotype		
	MM	MV	VV
1	10	3	4
2A	2	3	5
2B	10	-	-

Figure 3.2: Western blot analysis of human brain inocula transmitted to mice.

Brain inocula are shown in the middle lane and were run between sCJD MM1 (T1) and vCJD (T2B) diagnostic reference standards. Tissue extracts were PK digested and blots probed with the anti-PrP antibody 6H4. **(a)** Representative blots of inocula from all six subtypes of sCJD as classified according to *PRNP* codon 129 genotype and PrP^{res} type (Parchi et al., 1999). **(b)** Representative Western blots of inoculum from a single vCJD (CJD 31) case. All vCJD brain tissue showed the characteristic predominance of the diglycosylated form of PrP^{res} after Western blotting.

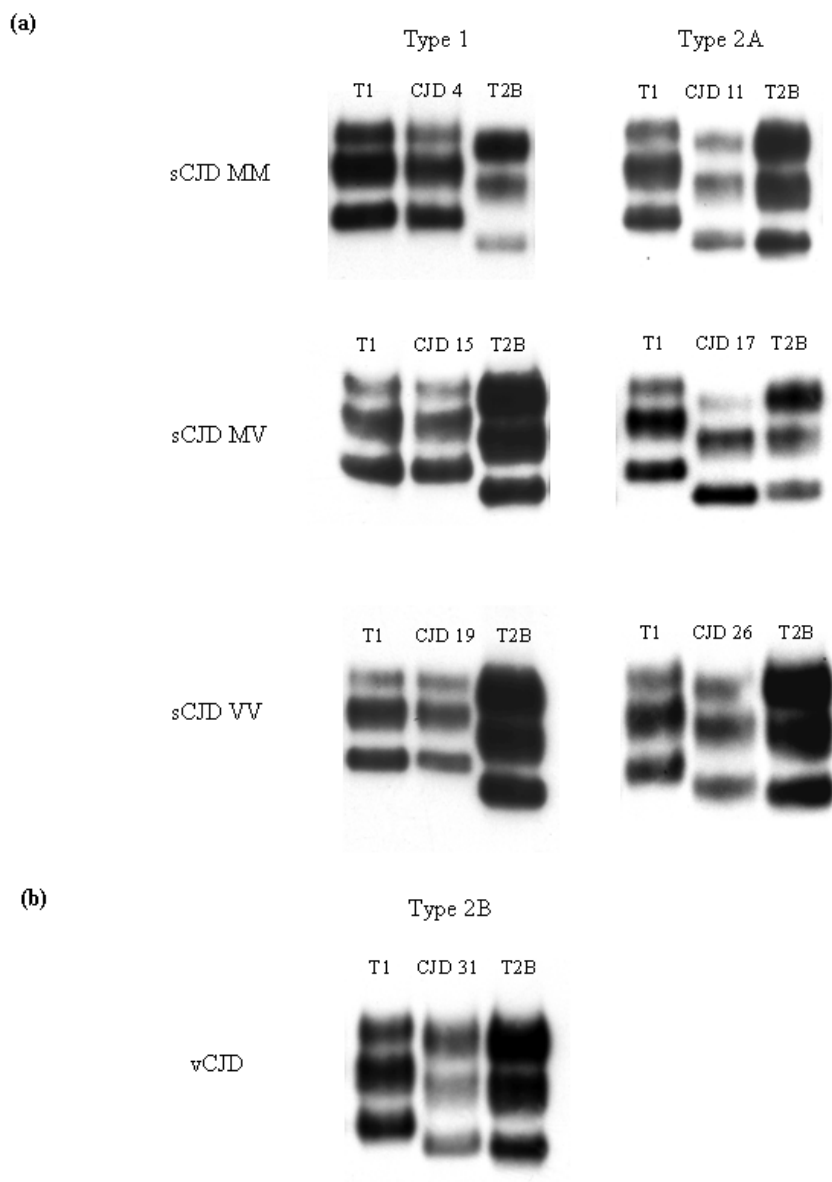


Figure 3.3: Neuropathology in frontal cortex tissue from patient CJD 11

(a) Haematoxylin and eosin labelling showing confluent spongiform most commonly associated with the MM2 subtype of sCJD. In addition, the presence of microvacuolar spongiform lesions most commonly associated with sCJD MM1 patients, are also evident. (b) PrP labelling with the anti-PrP antibody KG9 in the frontal cortex showing intense peri-vacuolar deposits of PrP most commonly associated with sCJD MM2 patients. A background of synaptic PrP labelling, a pathological feature sCJD MM1 cases is also observed. Original magnification x200.

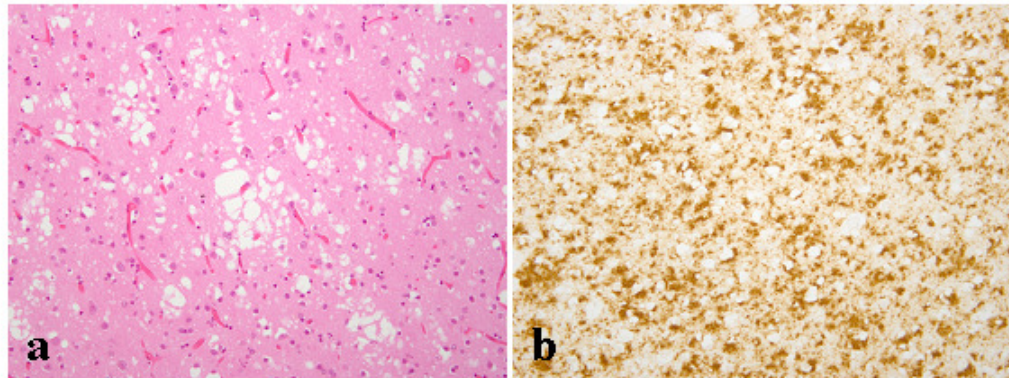
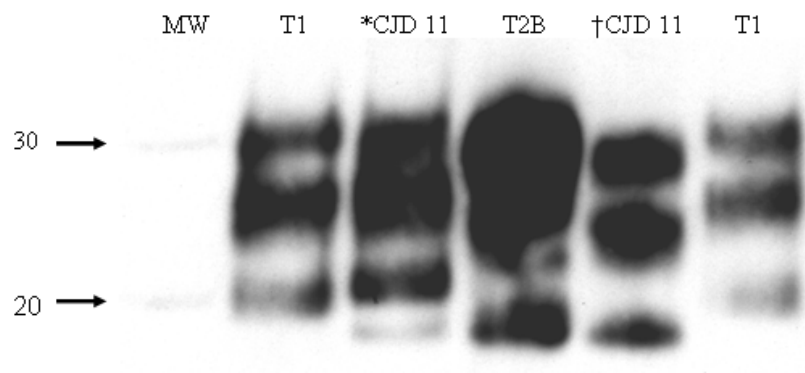


Figure 3.4: Western blot analyses of PrP^{res} types in brain extracts from CJD 11

Analysis of aliquots of the original frontal cortex sample analysed at diagnosis (*) showing the presence of PrP^{res} types 1 and a small amount of type 2. In contrast, an extract of inoculum (†) transmitted in the wild-type mouse panel, shows only PrP^{res} with a type 2 mobility. Samples shown are run between a sCJD MM1 (T1) and a vCJD (T2B) diagnostic reference standard. Tissue extracts were PK digested and blots probed with the anti-PrP antibody 6H4. Molecular weight markers (MW) are shown in kDa.



3.3.2 Detection of PrP^{res} in inoculum prepared from CJD lymphoid tissues, peripheral neural tissues and biological fluids

In order to determine the presence of infectivity within tissues other than brain, 41 transmission experiments were carried out on a number of lymphoid tissues (appendix, lymph node, spleen and tonsil), peripheral nervous system tissues (DRG, TRG and peripheral nerve), bone marrow and biological fluids and their constituent cells (buffy coat, plasma and cerebrospinal fluid) from four sCJD cases and seven vCJD cases (Table 3.3). Western blot analysis of peripheral tissues of CJD patients have demonstrated that the biochemical profile of PrP^{res} is subject to tissue-specific modifications (Glatzel et al. 2003; Head et al. 2004b; Peden et al. 2006; Peden et al. 2007). However, in the Western blot analysis of inocula used in this series of transmissions from peripheral tissues of CJD patients, the objective was not to carry out PrP^{res} isotyping but simply to establish whether PrP^{res} was present at detectable levels in the samples inoculated into the mice. Due to the heterogeneous distribution of PrP^{Sc} in peripheral tissues and the valuable nature of these tissues, in the absence of any residual inocula, tissues were not re-sampled for further Western blot analysis.

Residual inoculum was available from six of the 41 transmission experiments (Table 3.3). These six different inocula were homogenates of lymphoid tissues and a single TRG. Previous bioassay studies have shown that infectivity levels in vCJD spleen and tonsil are significantly lower than those observed in brain (Bruce et al., 2001). In order to increase the sensitivity of the Western blot assay, an additional centrifugal concentration step was carried using the residual volume of each inoculum (Head et al., 2004b). Samples were run alongside an extract of tonsil from a non TSE-related neurodegenerative disorder and the same control tonsil sample spiked with a 1µl volume of the vCJD diagnostic reference standard. These samples were included as a positive control to confirm that PrP^{res} can be recovered from tonsil tissue using the centrifugation procedure. The volume of inocula analysed from each source and the results from the Western blot analysis are detailed in Table 3.3.

Table 3.3: PrP^{res} detection in tissue homogenates used in the transmission study

Case	Diagnosis	Tissue type transmitted	Volume of inoculum tested (µl)	PrP ^{res} +ve/-ve
CJD 10	sCJD	SPL BC	200 n/t	-ve -
CJD 11	sCJD	BC PL	n/t n/t	- -
CJD 15	sCJD	BC	n/t	-
CJD 18	sCJD	BC PL	n/t n/t	- -
CJD 31	vCJD	SPL TONS BC PL	n/a 50 n/t n/t	- -ve - -
CJD 35	vCJD	TONS BC PL	200 n/t n/t	+ve - -
CJD 36	vCJD	BC PL	n/t n/t	- -
CJD 37	vCJD	BC PL	n/t n/t	- -
CJD 38	vCJD	APP BM CSF DRG LN PN SC TRG	200 n/t n/t n/a n/a n/a n/a 100	-ve - - - - - - -ve
CJD 39	vCJD	APP BM DRG LN PN SC TRG	n/a n/t n/a n/a n/a n/a n/a	- - - - - - -
CJD 40	vCJD	APP BM CSF DRG LN PN SC TRG	200 n/a n/a n/a n/a n/a n/a n/a	-ve - - - - - - -

APP – appendix, BC – buffy coat, BM – bone marrow, CSF – cerebrospinal fluid, DRG – dorsal root ganglia, LN – lymph node, PL – plasma, PN – peripheral nerve, SC – spinal cord, SPL – spleen, TONS – tonsil, TRG – trigeminal ganglia

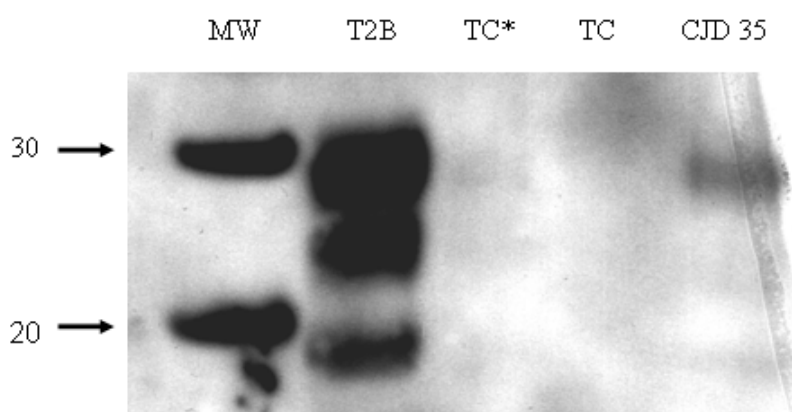
n/a – not available for analysis

n/t – not tested

PrP^{res} was detected in only a single inoculum tested. This single extract originated from a 200µl sample of tonsil homogenate from a vCJD case (CJD 35). The resulting PrP^{res} banding pattern of this tonsil extract was less intense than that of the 5µl vCJD brain standard (Figure 3.5), indicating that PrP^{res} levels were more than 40 times lower in this vCJD tonsil tissue than those found in vCJD brain. Although the PrP^{res} banding on the tonsil inocula extract was faint, the dominance of the diglycosylated fragment, which is characteristic of vCJD PrP^{res}, was clearly observed. No detectable levels of PrP^{res} were found following Western blot analysis of a 50µl tonsil extract from a second vCJD case (CJD 31). The remaining four inocula (sCJD spleen, vCJD appendix and vCJD TRG) analysed showed no detectable levels of PrP^{res} by Western blot analysis under these conditions (Table 3.3)

Figure 3.5: Western blot analyses of PrP^{res} in vCJD tonsil inocula

Blot shows PrP^{res} in an extract of inoculum (CJD 35) used in the experimental transmission to mice. This inoculum sample was prepared from tonsil tissue sampled from patient CJD 35. The inoculum was run alongside a vCJD (T2B) diagnostic reference standard, an extract of tonsil tissue taken from a patient with a non-TSE diagnosis (TC) and an extract of the same tonsil extract spiked with a 1µl vCJD of the vCJD diagnostic standard (TC*). Tissue extracts were PK digested and blots probed with the anti PrP antibody 6H4. Molecular weight markers (MW) are shown in kDa.



3.4 Results summary

Determining the PrP^{res} type of CJD brain inocula is fundamental to the interpretation of results from any transmission series. This study has confirmed;

- PrP^{res} was readily detected in all inocula prepared from sCJD and vCJD brain tissue
- Western blot analysis confirmed the inoculation of brain inocula from all six subgroups of sCJD as classified according to *PRNP* codon 129 and PrP^{res} type
- Re-analysis of one of the sCJD inocula corrected the predominant PrP type from type 1 to predominantly type 2A. The brain in this case is now recognised as containing both PrP^{res} types and has been sub-classified as MM1+2A
- PrP^{res} was detected in only one out of seven peripheral tissue homogenates used in the inoculation of mice

3.5 Discussion

This chapter had two specific aims. Firstly, to confirm the presence of PrP^{res} within tissue homogenates used in the inoculation of mice and secondly, to carry out PrP^{res} isotyping on brain homogenates used in the experimental transmission series. Using conventional Western blot analysis techniques, PrP^{res} was readily detected in all CJD brain homogenate analysed. Furthermore, PrP^{res} typing of individual brain inocula confirmed the transmission of brain homogenate from all six possible subgroups of sCJD as classified according to the *PRNP* codon 129 genotype and PrP^{res} type (Parchi et al., 1999). However, Western blot analysis on a limited number of peripheral neural and lymphoid tissue homogenates produced more variable results with only a single vCJD tonsil homogenate demonstrating detectable levels of PrP^{res}. PrP^{res} was not detected in three other vCJD lymphoid tissue homogenates or a single vCJD TRG homogenate, even though PrP^{Sc} deposition in these tissues had been confirmed after immunohistochemical analysis of paraffin fixed tissue sections. Furthermore, PrP^{res} was not detected in any inocula prepared from sCJD spleen tissue.

3.5.1 Infectivity levels within peripheral tissues

Western blotting on extracts of CJD brain inocula used in the transmission series (Figure 3.2) demonstrated a more intense PrP^{res} banding pattern when compared to that of the vCJD tonsil inocula (Figure 3.5), even though volumes of brain inocula tested were 40 times lower than that analysed in the tonsil extract. These observations are consistent with bioassay experiments which have indicated that infectivity levels in vCJD spleen and tonsil tissue are significantly lower than those demonstrated in vCJD brain (Bruce et al., 2001).

The presence of readily detectable levels of PrP^{Sc} in peripheral tissues is not a general feature of sCJD (Head et al., 2004b). In this study, the sCJD spleen tissue sampled for transmission to mice had shown no evidence of PrP^{Sc} by immunohistochemical analysis for the prion protein. Therefore, the lack of any detectable PrP^{res} in these tissue homogenates after Western blotting was not unexpected. In contrast, PrP^{Sc} had been demonstrated by immunohistochemistry in

all vCJD lymphoid tissues and peripheral neural tissues selected for transmission and so the lack of any detectable PrP^{res} in four of the five tissue homogenates tested was somewhat surprising. One possible explanation may be simply a sampling problem. In the preparation of inocula for transmission, all tissues were re-sampled, raising the possibility that infectivity was present in these tissues, but that the small samples taken had missed the major sites of PrP^{Sc} deposition, perhaps due to the heterogeneous distribution of PrP^{Sc} in these tissues. Another possible explanation for the lack of any detectable PrP^{res} in the vCJD homogenate could lie with the sensitivity of the Western blot assay. It may be that PrP^{Sc} was present within the four tissue homogenates but at levels which are below the sensitivity of conventional Western blotting techniques, even after centrifugal concentration. This appears to be the most likely explanation as experimental challenge with the vCJD tonsil (CJD 31), TRG (CJD 38) and appendix (CJD 38) homogenates that had shown no detectable levels of PrP^{res} after Western blotting; all produced either clinical and/or pathological evidence of a TSE in a proportion of mice challenged. This confirms the presence of infectivity in these tissue homogenates. However, a further explanation which cannot be discounted is that the long term storage of these tissue homogenates has resulted in the degradation or insolubility of PrP^{Sc} present in the inoculum samples. The results for the experimental challenge of different CJD tissue types are discussed in detail in chapter 6.

3.5.2 Detection of PrP^{Sc} in peripheral tissues in CJD

Conventional Western blotting on the different tissue inocula has demonstrated that this method is sufficiently sensitive to detect PrP^{res} in post-mortem brain tissue from CJD patients at the end stage of disease. However, Western blotting is insufficiently sensitive for the reliable detection of PrP^{Sc} in lymphoid tissues and peripheral neural tissues when compared to bioassay in mice. Although sensitive, bioassay experiments are long and expensive and do not provide a practical solution for the routine detection of PrP^{Sc} in suspected cases of CJD or in the development of a diagnostic test for the presence of PrP^{Sc} in biological solutions such as blood and urine. The recent development of techniques such as conformation dependant immunoassay (CDI) (Safar et al., 1998), sodium phosphotungstate (NaPTA)

precipitation (Safar et al., 1998; Wadsworth et al., 2001) and protein misfolding cyclic amplification (PMCA) (Saborio et al., 2001; Castilla et al., 2005) address these issues and have been developed specifically to increase the sensitivity of the assay. It would be interesting to see whether PrP^{Sc} can be detected in the vCJD tissue homogenates using any of these methods, thus allowing a direct comparison between these new innovative methods with that of traditional bioassay in mice.

3.6 Chapter conclusion

The characterisation of inocula used in the transmission study has demonstrated that Western blot analysis is a reliable and sensitive method for the detection and typing of PrP^{res} in extracts of CJD brain homogenate and has largely confirmed the biochemical type of PrP^{res} involved. However, the sensitivity of the Western blot assay may be below the limits for the detection of PrP^{res} in lymphoid and peripheral neural tissues or the samples may no longer be suitable for this form of analysis.

Chapter 4: Characterisation of the sporadic Creutzfeldt-Jakob disease agent by transmission to wild-type mice

4.1 Objective

1. To characterise the sCJD agent strain after primary transmission of brain tissue in panels of wild-type type mice using the standard strain typing properties of incubation period and lesion profiling, plus a full analysis of PrP in the mouse brain and analysis of the PrP^{res} molecular subtypes present.
2. To further extend the analysis of the sCJD agent strain after subsequent mouse-to-mouse passages.

4.2 Introduction

A number of distinct strains of scrapie in sheep have been identified following experimental transmission to inbred lines of mice. These strains are distinguished by the observation of simple disease characteristics, specifically incubation period and patterns of TSE-specific vacuolar pathology (“lesion profile”) in the brain after serial passage through inbred lines of mice. These same strain typing methods were instrumental in providing early evidence for the close similarity between the BSE agent and that of vCJD, while confirming that the vCJD agent represented a new human TSE distinct from that which causes sCJD (Bruce et al., 1997). However, it has yet to be established whether there are distinct strains of agent which are responsible for the phenotypic heterogeneity that is observed sCJD.

According to the prion hypothesis, strain-specific biological properties are enciphered in the different conformations and glycosylation states of PrP^{Sc} (Collinge et al., 1996; Telling et al., 1996). Molecular typing of PrP^{res} in the brain of sCJD patients by Western blot analysis has shown that the abnormal protein can exist in different conformational states distinguished by differences in the extent of their N-terminal truncation following partial protease digestion (Parchi et al., 1996; Hill et al., 2003). These different conformational states or PrP^{res} types, in combination with the M/V polymorphism at codon 129 on the *PRNP* gene, have been proposed as the major determinants of the different clinico-pathological phenotypes in sCJD and

form the basis of two different classification systems for sCJD; that outlined by Collinge and co-workers (Collinge et al., 1996; Wadsworth et al., 1999a; Wadsworth et al., 1999b; Hill et al., 2003) and that of Parchi and Gambetti (Parchi et al., 1996; Parchi et al., 1997; Parchi et al., 1999; Hill et al., 2003). Both proposed classification systems are described in detail in Chapter 1, section 1.10.

This thesis follows the classification system described by Parchi and Gambetti, in which two major PrP^{res} types are identified in sCJD; type 1 where the molecular weight of the unglycosylated fragment is approximately 21kDa and type 2 with a molecular weight of the unglycosylated fragment of approximately 19kDa (Parchi et al., 1996; Parchi et al., 1999; Hill et al., 2003). In this proposed classification system, six different subtypes of sCJD have been described based on differing clinico-pathological phenotypes (Parchi et al., 1999). Each of these six phenotypic subtypes closely correlates with the different combinations of *PRNP* codon 129 genotype and PrP^{res} type. However, the question of whether these different sCJD subtypes relate to different TSE strains remains an unsubstantiated assumption.

Molecular isotyping of PrP^{res} is increasingly used as a surrogate marker for agent strain. In human TSEs, differences in PrP^{res} type have been used as a diagnostic tool to distinguish sCJD from vCJD (Collinge et al., 1996). In their original study on the classification of sCJD, Parchi and Gambetti reported the co-occurrence of PrP^{res} types 1 and type 2 in the brain in around 5% of sCJD cases examined (Parchi et al., 1999). Several studies have since concurred with Parchi and Gambetti's findings and estimate that co-occurrence of PrP^{res} types is a feature of between 5% and 30% of sCJD cases (Collinge et al., 1996; Puoti et al., 1999; Kovacs et al., 2002a; Hill et al., 2003; Haik et al., 2004; Head et al., 2004a; Schoch et al., 2006; Uro-Coste et al., 2008). In contrast, two studies using monoclonal antibodies specifically raised against PrP^{res} type 1 suggest that the co-occurrence of PrP^{res} types in sCJD cases is underestimated and is much higher than initially thought (Polymenidou et al., 2005; Yull et al., 2006; Yull et al., 2009). Regardless of the percentage of sCJD in which there is the co-occurrence of PrP^{res} types, this feature of sCJD has cast some doubt over the validity of molecular classification of sCJD and the relationship between

PrP^{res} types and molecular strain. More recently the question of whether the co-occurrence of PrP^{res} type 1 and type 2 in the brain of sCJD cases results in a distinct phenotype has caused the original classification system for sCJD to be re-addressed creating additional sCJD clinico-pathological subtypes in the 30-40% of cases that show some degree of co-occurrence (Parchi et al., 2009; Cali et al., 2009). This thesis predates this proposed re-classification of sCJD and has addressed the co-occurrence issue in the form of direct testing of inocula for their PrP^{res} type rather than relying on homogeneity throughout the brain (see chapter 3)

This particular chapter addresses the question of whether distinct isolates or TSE strains are present within sCJD. The vast majority of information on the sCJD agent has come from the transmission of sCJD in numerous transgenic mouse lines over expressing human PrP or in chimeric human-mouse PrP mice (Telling et al., 1994; Korth et al., 2003; Taguchi et al., 2003; Asante et al., 2006; Kobayashi et al., 2007; Wadsworth et al., 2008; Bishop et al., 2010). Transmission of sCJD into bank voles has also provided a valuable insight into the existence of different sCJD strains (Nonno et al., 2006). However, at present it remains that strain typing is most highly developed using transmission into panels of non-transgenic (wild-type) mice. Previous studies examining the transmission properties of sCJD to wild-type mice suggested that a full characterisation of the sCJD agent is hampered by the ‘species barrier’ effect between the donor and recipient (Brown et al., 1994; Bruce et al., 1997). A limitation of these studies is that a full and direct comparison of the strain typing properties of the sCJD agent present in each of the six sCJD subtypes as classified according to Parchi’s 1999 classification system has not been examined. In response to this, this chapter provides a full and direct characterisation of the sCJD agent following inoculation of panels of wild-type mice with brain homogenates from sCJD cases of all six possible combinations of *PRNP* codon 129 genotype and PrP^{res} type. As a result of the findings from studies on the co-occurrence of PrP^{res} types in sCJD, an important aspect in the accurate interpretation of results from this transmission series was to establish the actual PrP^{res} type in the homogenate used to inoculate the mice. Therefore, Western blot analysis on inocula from each sCJD source was carried out as described in detail in chapter 3.

Results from these primary transmissions will directly test the relationship between the biochemical properties of the prion protein (PrP^{res} type) and M/V polymorphism at codon 129 with the biological properties of the agent (i.e. strain). Consequently, the results can be used to question whether the clinico-pathological phenotypes in sCJD as described by Parchi and Gambetti do indeed correlate with distinct strains of TSE agent.

4.3 Experimental results: Primary transmissions

Brain homogenates from 27 cases of clinically and neuropathologically confirmed sCJD (Chapter 2, Table 2.1; CJD 1 – CJD 27) were experimentally inoculated into panels of wild-type mice (RIII, C57BL, VM and C57BL x VM). Brain homogenates were prepared from all six possible combinations of *PRNP* codon 129 genotype and PrP^{res} type (type 1 or type 2A). Throughout this thesis, the term “subgroup” is used in reference to sCJD cases classified according to their *PRNP* codon 129 genotype/PrP^{res} type combination. The term “subtype” refers to the classification of sCJD cases according to their clinical and pathological features as described by Parchi and Gambetti (Parchi et al., 1999). Brain homogenates from 25 of the sCJD cases were prepared from grey matter tissue of the frontal cortex, with the remaining two brain homogenates prepared from tissue taken from the cerebellar hemisphere. Inoculations were carried out either via a single IC injection or a combination of the IC and IP routes. Brain region used in inoculum preparation, inoculation routes and mouse lines challenged for each sCJD brain isolate are detailed in Chapter 2, Table 2.3.

4.3.1 Incubation periods and incidence of disease

In any transmission experiment, intercurrent illness and death reduces the numbers of mice available for strain typing analysis. In this transmission series, only mice surviving over 250dpi were included in the analysis. Mean incubation periods were drawn from experimental groups with at least five mice that had received a positive score for clinical signs of disease, with disease confirmed histologically having received a positive score for the presence of TSE-associated vacuolar degeneration in the brain.

4.3.1.1 sCJD MM1 subgroup

Ten transmission experiments were carried out with brain tissue from 10 patients who were homozygous for methionine at codon 129 of the *PRNP* gene and who were confirmed as having a PrP^{res} type 1 after Western blot analysis of the inocula. Table 4.1 details the incidence of clinical signs of disease and vacuolar pathology in each experimental group. Clinical signs of a neurological disease in mice challenged with brain homogenate from sCJD MM1 cases were rare. Only 12 mice (eight C57BL mice and four VM mice) from the 580 surviving over 250dpi received a positive score for clinical signs of TSE disease, with disease confirmed by the presence of vacuolar degeneration typical of TSE infection seen in the brains of the mice at post-mortem (Table 4.1). All mice that developed clinical signs of a TSE had lengthy incubation periods of over 650 days. A few mice (4/580) received a positive score for clinical signs of TSE disease but had a negative score for vacuolar pathology after histological analysis (Table 4.1). Assessing clinical signs of a TSE in mice is challenging and involves a great deal of training. Nevertheless in such an extensive transmission series as this it is not unexpected to encounter a small number of mice scored as positive for clinical signs of disease but which are then found to be negative for TSE pathology. This is, particularly true for very old mice. While the reason for the discordance between clinical signs and pathological confirmation cannot be known with certainty, it is the working assumption of this thesis that the neuropathological assessment is definitive and that in these few examples the positive clinical signs are either in doubt or indicative of an unrelated illness or ageing.

The lack of any clinical signs of neurological disease in the majority of mice does not however indicate a failure to transmit sCJD from the MM1 brain homogenates, as vacuolar degeneration was observed in the brains of 424 out of 580 mice of mice dying with intercurrent disease (Table 4.1). A full analysis of the vacuolation in the mouse brain will be discussed in detail in section 4.3.2. No clinical signs of disease or vacuolar degeneration were found in the control mice of any age in this series of transmissions (data not shown).

Table 4.1: Primary transmission of sCJD MM1 brain homogenate

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice challenged with brain tissue from ten sporadic CJD cases, homozygous for methionine at codon 129 of the *PRNP* gene with a PrP^{res} type 1. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Mouse line							
	C57BL		RIII		VM		C57BL x VM	
Source	Clin	Vac path	Clin	Vac path	Clin	Vac path	Clin	Vac path
*CJD 1	0/8	6/8	0/15	7/15	0/19	16/19	0/16	10/16
†CJD 2	0/9	5/9	0/13	11/13	0/16	14/16	0/17	12/17
*CJD 3	0/12	4/12	0/9	5/9	0/16	15/16	0/15	13/15
*CJD 4	1/9 (path –ve)	6/9	0/9	7/9	0/16	16/16	0/16	15/16
*CJD 5	0/9	3/9	0/15	9/15	0/14	14/14	0/19	17/19
*CJD 6	0/15	8/15	0/14	9/14	0/14	11/14	0/15	10/15
*CJD 7	4/24 (833, 834, 864, path –ve)	12/24	0/25	9/25	0/18	13/18	0/28	17/28
*CJD 8	1/12 (755)	12/12	0/15	15/15	0/22	22/22	0/16	15/16
*CJD 9	4/15 (652, 690, 692, 698)	9/15	0/22	14/22	2/18 (694, 726)	18/18	0/20	16/20
‡CJD 10	nd	nd	1/6 (path –ve)	3/6	3/9 (699, 735, path –ve)	6/9	nd	nd

* Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from frontal cortex tissue

† Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from cerebellar cortex tissue

‡ Intracerebral injection with 10% brain inoculum prepared from frontal cortex tissue

nd Not done

4.3.1.2 sCJD MV1 subgroup

Three transmission experiments were carried out with brain tissue from three patients who were heterozygous for methionine and valine at codon 129 of the *PRNP* gene and who were confirmed as having a PrP^{res} type 1 after Western blot analysis of the inocula. Table 4.2 details the incidence of clinical signs of disease and vacuolar pathology in each experimental group. No clinical signs of neurological disease related to a TSE were confirmed in the mice (clinical signs were observed in a single RIII mouse but histological analysis of the brain failed to show the presence of vacuolar degeneration). Therefore, incubation period was not a relevant parameter in the strain typing analyses of sCJD MV1 brain homogenate. As found with sCJD MM1 brain homogenate, the absence of clinical signs in the sCJD MV1 inoculated mice does not indicate a failure to transmit disease, as vacuolar degeneration was observed in the brains of 109 out of 166 inoculated mice (Table 4.2). No clinical signs of disease or vacuolar degeneration were found in the control mice of any age in this series of transmissions (data not shown).

4.3.1.3 sCJD VV1 subgroup

Inoculation of brain homogenate from four patients who were homozygous for valine at codon 129 of the *PRNP* gene and who were confirmed as having a PrP^{res} type 1 after Western blot analysis of the residual inocula was carried out in the mouse panel. Table 4.3 details the incidence of clinical signs of disease and vacuolar pathology in each experimental group. No clinical signs of neurological disease related to a TSE were confirmed in the mice (clinical signs were observed in a single RIII mouse but histological analysis failed to show the presence of vacuolar degeneration). Therefore, incubation period was not a relevant parameter in the strain typing analyses of sCJD VV1 brain homogenates. In contrast to transmissions from sCJD MM1 and sCJD MV1 brain homogenate, histological examination of the mouse brain showed vacuolar degeneration associated with TSE infection was rare, even in mice surviving over 800 days after challenge. Only a single C57BL x VM mouse produced vacuolar pathology (survival time 915 days after challenge) (Table 4.3). This clinical and histological data suggests that transmission of sCJD from the VV1 subgroup is rare in wild-type mice.

Table 4.2: Primary transmission of sCJD MV1 brain homogenate

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice challenged with brain tissue from three sporadic CJD cases, heterozygous for methionine and valine at codon 129 of the *PRNP* gene with a PrP^{res} type 1. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Mouse line							
	C57BL		RIII		VM		C57BL x VM	
Source	Clin	Vac path	Clin	Vac path	Clin	Vac path	Clin	Vac path
*CJD 13	0/11	6/11	0/15	10/15	0/21	19/21	0/17	15/17
†CJD 14	0/15	5/15	1/17 (path –ve)	9/17	0/18	11/18	0/20	11/20
*CJD 15	nd	nd	0/15	11/15	0/17	12/17	nd	nd

* Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from frontal cortex tissue

† Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from cerebellar cortex tissue

nd Not done

Table 4.3: Primary transmission of sCJD VV1 brain homogenate

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice challenged with brain tissue from four sporadic CJD cases, homozygous for valine at codon 129 of the *PRNP* gene with a PrP^{res} type 1. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Mouse line							
	C57BL		RIII		VM		C57BL x VM	
Source	Clin	Vac path	Clin	Vac path	Clin	Vac path	Clin	Vac path
*CJD 19	0/18	0/18	1/22 (path –ve)	0/22	0/26	0/26	0/30	0/30
‡CJD 20	0/17	0/17	0/23	0/23	0/22	0/22	0/19	0/19
*CJD 21	0/9	0/9	0/11	0/11	0/13	0/13	0/11	1/11
*CJD 22	0/14	0/14	nd	nd	nd	nd	nd	nd

* Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from frontal cortex tissue

‡ Intracerebral injection with 10% brain inoculum prepared from frontal cortex tissue

nd Not done

4.3.1.4 sCJD MM2A subgroup

Inoculation of brain homogenate from two sCJD patients who were homozygous for methionine at codon 129 of the *PRNP* gene and who were confirmed as having a PrP^{res} type 2 after Western blot analysis of inocula was carried out in the mouse panel. Table 4.4 details the incidence of clinical and pathological signs in each experimental group. In contrast to all other sCJD subgroups investigated, seven out of 19 RIII mice inoculated with brain homogenate from one MM2 case (patient CJD 12) were sacrificed with clinical TSE disease, allowing a mean incubation period of 736 ± 9 days to be calculated for this experimental group. The development of clinical TSE disease in this RIII group distinguishes this sCJD MM2 case from the other 26 sCJD cases examined. Clinical TSE disease was not observed in any other mouse line following inoculation with the same CJD isolate. In contrast, inoculation of brain homogenate from a second sCJD MM2 case (patient CJD 11) showed no clinical TSE disease in any of the mice inoculated (clinical signs were observed in a single RIII mouse but histological analysis showed no evidence of vacuolar pathology). Such differences in the efficiency of transmission between these two sCJD MM2 brain homogenates may be indicative of differences in infectious titres between the two inocula. Alternatively, it may also be that in the primary transmission of brain homogenate from sCJD MM2, the IP route is more efficient than the IC route. Although somewhat surprising, this has been shown previously in the primary transmission of BSE (Bruce et al., 1994). Regardless of the differences between the inoculum, these experiments have shown that transmission of a TSE from sCJD MM2 brain homogenate, as judged by clinical and vacuolar pathology does occur. These findings also suggest that there may be subtle differences in the transmission properties of the sCJD MM2 subgroups compared to the other sCJD subgroups which can be distinguished after transmission in the RIII mouse line.

Table 4.4: Primary transmission of sCJD MM2A brain homogenate

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice challenged with brain tissue from two sporadic CJD cases, homozygous for methionine at codon 129 of the *PRNP* gene with a PrP^{res} type 2. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Mean incubation periods in days are shown in brackets \pm S.E.M. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Mouse line							
	C57BL		RIII		VM		C57BL x VM	
Source	Clin	Vac path	Clin	Vac path	Clin	Vac path	Clin	Vac path
‡CJD 11	nd	nd	1/24 (path –ve)	3/24	0/14	4/14	nd	nd
*CJD 12	nd	nd	7/19 (736 \pm 9)	12/19	0/20	2/20	0/14	4/14

* Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from frontal cortex tissue

‡ Intracerebral injection with 10% brain inoculum prepared from frontal cortex tissue

nd Not done

4.3.1.5 sCJD MV2A subgroup

Inoculations were carried out with brain tissue from three patients who were heterozygous for methionine and valine at codon 129 of the *PRNP* gene and who were confirmed as having a PrP^{res} type 2A after Western blot analysis of the brain homogenate. Table 4.5 details the incidence of clinical and pathological signs in each experimental group. No clinical signs of neurological disease related to a TSE were observed in the mice (clinical signs were observed in two RIII mice but histological analysis showed no evidence of vacuolar pathology). Therefore incubation period is not a relevant parameter in the strain typing analyses of sCJD from MV2 cases. As found with inoculations from sCJD VV1 brain homogenates, TSE associated vacuolar degeneration in the brain was rare, with only a single RIII mouse producing vacuolar pathology with a survival time of 711 days after challenge (Table 4.5). This data suggests that transmission of sCJD from the MV2 subgroup is a rare occurrence. No clinical signs of disease or vacuolar degeneration were found in the control mice of any age in this series of transmissions.

4.3.1.6 sCJD VV2A subgroup

Transmission of brain homogenate from five sCJD cases in which patients were homozygous for valine at codon 129 of the *PRNP* gene and who were confirmed as having a PrP^{res} type 2A after Western blot analysis of the inocula were carried out in the mouse panel. Table 4.6 details the incidence of clinical and pathological signs in each experimental group. No clinical signs of neurological disease related to a TSE were observed in the mice; therefore, incubation period is not a relevant parameter in the strain typing analyses of sCJD from the VV2 subgroup (clinical signs were observed in four mice; however, histological analysis failed to confirm a TSE). Vacuolar pathology in the mice was rare with only a single C57BL mouse showing vacuolar pathology with a survival time of 845 days after challenge (Table 4.6). These results suggest that successful transmission of TSE disease from the sCJD VV2 subgroup is rare.

Table 4.5: Primary transmission of sCJD MV2A brain homogenate

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice challenged with brain tissue from three sporadic CJD cases, heterozygous for methionine and valine at codon 129 of the *PRNP* gene with a PrP^{res} type 2. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Mouse line							
	C57BL		RIII		VM		C57BL x VM	
Source	Clin	Vac path	Clin	Vac path	Clin	Vac path	Clin	Vac path
*CJD 16	0/20	0/20	0/21	1/21	0/20	0/20	0/19	0/19
*CJD 17	0/17	0/17	0/18	0/18	0/20	0/20	0/15	0/15
‡CJD 18	nd	nd	2/7 (path –ve)	0/7	nd	nd	nd	nd

* Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from frontal cortex tissue

‡ Intracerebral injection with 10% brain inoculum prepared from frontal cortex tissue

nd Not done

Table 4.6: Primary transmission of sCJD VV2A brain homogenate

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice challenged with brain tissue from five sporadic CJD cases, homozygous for valine at codon 129 of the *PRNP* gene with a PrP^{res} type 2. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Mouse line							
	C57BL		RIII		VM		C57BL x VM	
Source	Clin	Vac path	Clin	Vac path	Clin	Vac Path	Clin	Vac path
*CJD 23	0/18	1/18	0/21	0/21	2/23 (path –ve)	0/23	0/23	0/23
*CJD 24	0/5	0/5	0/14	0/14	0/2	0/2	0/6	0/6
*CJD 25	2/22 (path –ve)	0/22	0/24	0/24	0/23	0/23	0/20	0/20
*CJD 26	0/22	0/22	0/24	0/24	0/23	0/23	0/19	0/19
*CJD 27	0/16	0/16	0/8	0/8	0/18	0/18	0/10	0/10

* Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from frontal cortex tissue

4.3.2 Patterns of vacuolar pathology- lesion profiles

The presence of vacuolar degeneration, typical of TSE infection within the mouse brain is required for the positive confirmation of the successful transmission of TSE isolates in mice, even in mice which have received a positive score for clinical signs of disease. When combined with incubation period patterns in different mouse genotypes, the lesion profile produces a “signature” of the biological agent, which can be used to characterise the TSE strain present in an isolate from a naturally infected host. The lesion profiling and its contribution as a strain typing parameter in TSE disease is described in detail in Chapter 1.

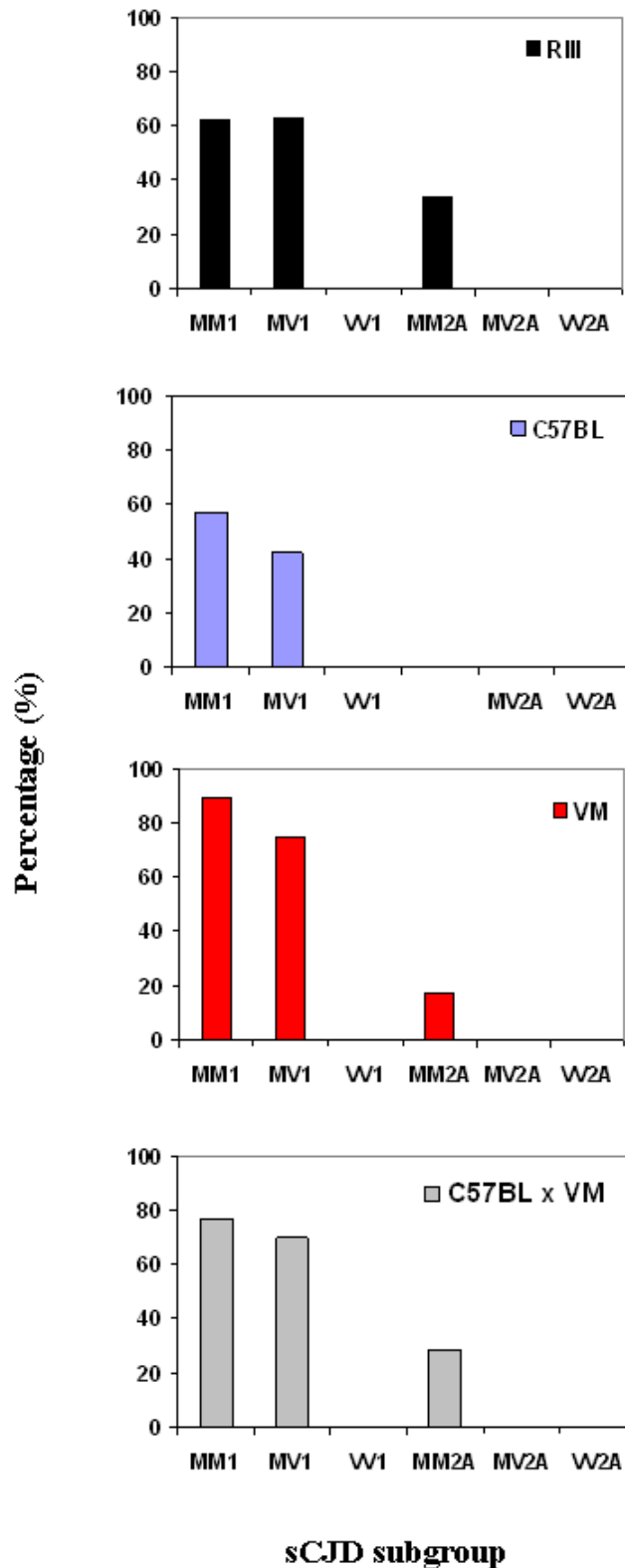
In the transmission of brain homogenate from all six sCJD subgroups into the mouse panel, clinical signs of a neurological disease consistent with a TSE in mice were rare (Table 4.1 – 4.6). However, this did not indicate a failure to transmit sCJD, as vacuolar degeneration typical of TSE disease was seen in the brains of a proportion of the mice dying with intercurrent illness (Table 4.1 – 4.6). Histological examination of the brains of all inoculated mice was carried out blind to the experimental details, the mouse line and the sCJD subgroup from which the inoculum was prepared. Scoring of vacuolar changes in the mouse brain and construction of the lesion profiles were carried out as described in chapter 2, section 2.6.3. Traditionally, lesion profiles are constructed from mice sacrificed at the clinical end-point of disease in which mice have received a positive score for clinical signs of a TSE and where disease was confirmed histologically. In these investigations on the transmission of sCJD, in the lack of mice sacrificed with signs of clinical TSE disease, lesion profiles were constructed from animals which had received a positive score for vacuolar degeneration in the brain and sacrificed with either intercurrent illness or at the full life span of the mouse.

TSE-specific vacuolation was most prominent in mice inoculated with brain homogenate from the sCJD MM1 (Table 4.1), MV1 (Table 4.2) and MM2 (Table 4.4) subgroups (Figure 4.1), with sufficient mice having received a positive score for vacuolar pathology for the construction of a lesion profile. In contrast, as only a single mouse challenged with brain homogenate from the sCJD MV2 (Table 4.5),

VV1 (Table 4.3) and VV2 (Table 4.6) subgroups received a positive score for TSE-specific vacuolation; there were insufficient mice available for the construction of a lesion profile. Lesion profiling is therefore not a relevant parameter in the strain typing of sCJD from MV2, VV1 and VV2 cases. Although it is not possible to compare the single mouse profiles from the sCJD MV2, VV1 and VV2 challenged mice, it is worth noting that analysis of these single mouse profiles showed a pattern and intensity of vacuolation consistent to that observed in mice inoculated with sCJD MM1 and MV1 brain homogenate. In particular this is characterised by a peak in the intensity of vacuolation in the superior colliculus (G3). Lesion profile data for the MM1, MV1 and MM2 subgroups of sCJD are detailed in the following sections.

Figure 4.1: TSE-specific vacuolar pathology in sCJD inoculated mice

Bar graph showing the percentage of mice receiving a positive score for TSE-specific vacuolar pathology following histological analysis of the brain. Results shown are the combined results from the individual brain homogenate from each sCJD subgroup.

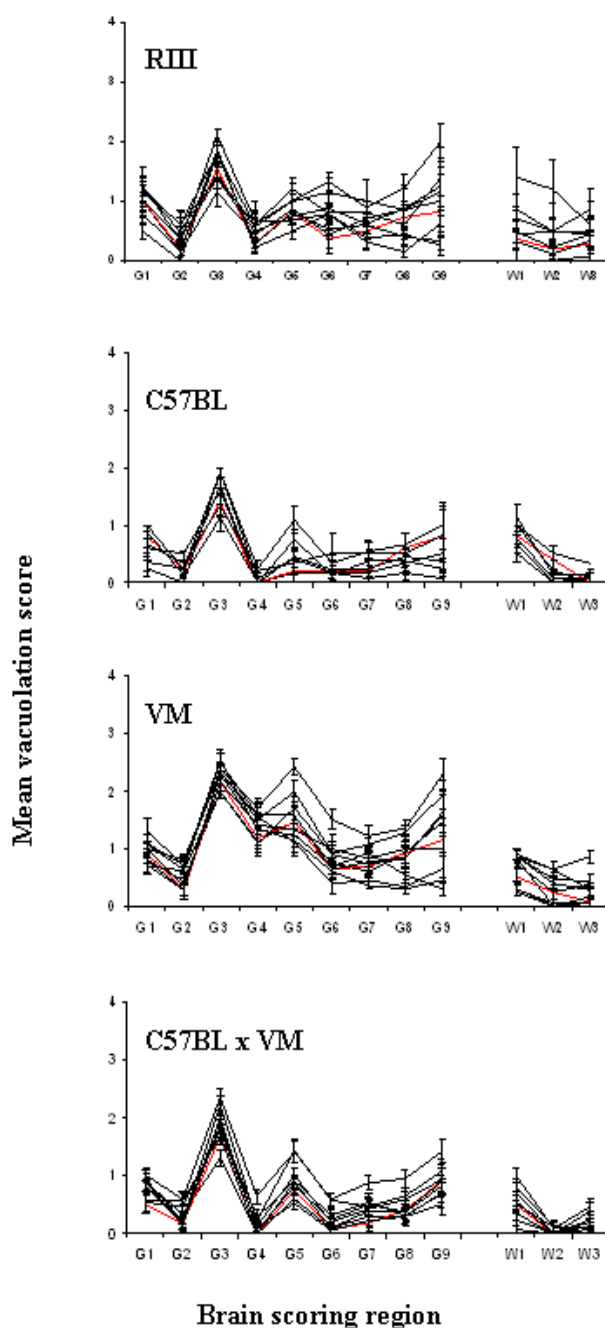


4.3.2.1 sCJD MM1 subgroup

All but three experimental groups (C57BL mice inoculated with CJD 3, C57BL mice inoculated with CJD 5, RIII mice inoculated with CJD 10) had sufficient mice scoring positive for TSE-specific vacuolation in the brain for the construction of a lesion profile. Mean lesion profiles in each mouse line are shown in Figure 4.2. Overall, patterns of vacuolar pathology were remarkably similar between individual sCJD cases and between the different mouse *Prn-p* genotypes, regardless of brain region used in the inoculation or route of injection. There was some variability in the severity of vacuolation between individual sCJD brain homogenates, most notable in the VM mouse line; however the general pattern of vacuolation was similar. Lesion profiles are characterised by a distinct and consistent peak of mild-to-moderate vacuolation targeting the superior colliculus (brain scoring region G3). Vacuolation, although less severe, is also prominent in the thalamus (scoring region G5) and the cerebral cortex (scoring region G8 and G9). Although these profiles are not based on mice at clinical end-point of disease, they clearly show a similarity in the lesion profile between the 10 sCJD MM1 brain homogenates and between the different mouse *Prn-p* genotypes.

Figure 4.2: Lesion profiles for sCJD MM1 inoculated mice

Lesion profiles are constructed for groups in which there was a minimum of five mice with a positive score for TSE associated vacuolar pathology. Vacuolation was scored at nine specified grey (G) matter regions and three white (W) matter regions of the mouse brain. Lesion profiles are shown with standard error bars (\pm S.E.M). Each line represents a different sCJD MM1 case. (black line) brain homogenate prepared from frontal cortex tissue, (red line) brain homogenate prepared from the cerebellar cortex. No distinction is made between different inoculation routes.

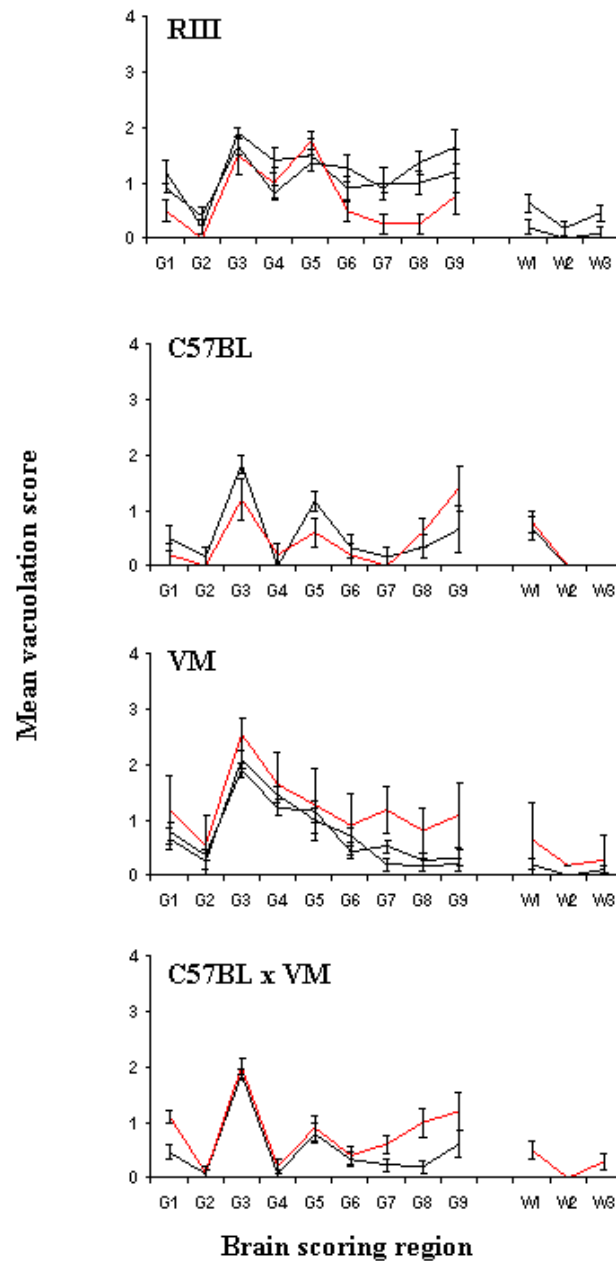


4.3.2.2 sCJD MV1 subgroup

Sufficient mice receiving a positive score for the presence of TSE-specific vacuolation in the brain were available in all experimental groups inoculated with sCJD MV1 brain homogenate for the construction of a lesion profile (Figure 4.3). As observed in sCJD MM1 inoculated mice, there was a consistent targeting of mild-moderate vacuolation in the superior colliculus (brain scoring region G3) in all four mouse lines. Differences in the severity of vacuolation were observed between the RIII, C57BL and C57BL x VM mouse lines between scoring regions G5 (thalamus) and G9 (cerebral cortex); however, the general pattern of vacuolation was similar in these three mouse lines. Subtle differences in the pattern of vacuolar pathology were observed in the VM mice, most obviously in the thalamus region (brain scoring region G5) where there was no obvious peak in vacuolar degeneration compared to the other mouse lines. The brain region used in inoculation and route of inoculation had no observable effect on the targeting of vacuolar pathology. These lesion profiles are not based on mice at the clinical end-point of disease; therefore, some variation in vacuolar degeneration would be expected from mice sacrificed at different stages in the disease progression. However, they clearly show a similarity in lesion distribution between the three MV1 brain homogenates and show that minor differences were observed in the vacuolar pathology between the different mouse *Prn-p* genotypes.

Figure 4.3: Lesion profiles for sCJD MV1 inoculated mice

Lesion profiles are constructed for groups in which there was a minimum of five mice with a positive score for TSE associated vacuolar pathology. Vacuolation was scored at nine specified grey (G) matter regions and three white (W) matter regions of the mouse brain. Lesion profiles are shown with standard error bars (\pm S.E.M). Each line represents a different sCJD MV1 case. (black line) brain homogenate prepared from frontal cortex tissue, (red line) brain homogenate prepared from the cerebellar cortex. No distinction is made between different inoculation routes



4.3.2.3 sCJD MM2A subgroup

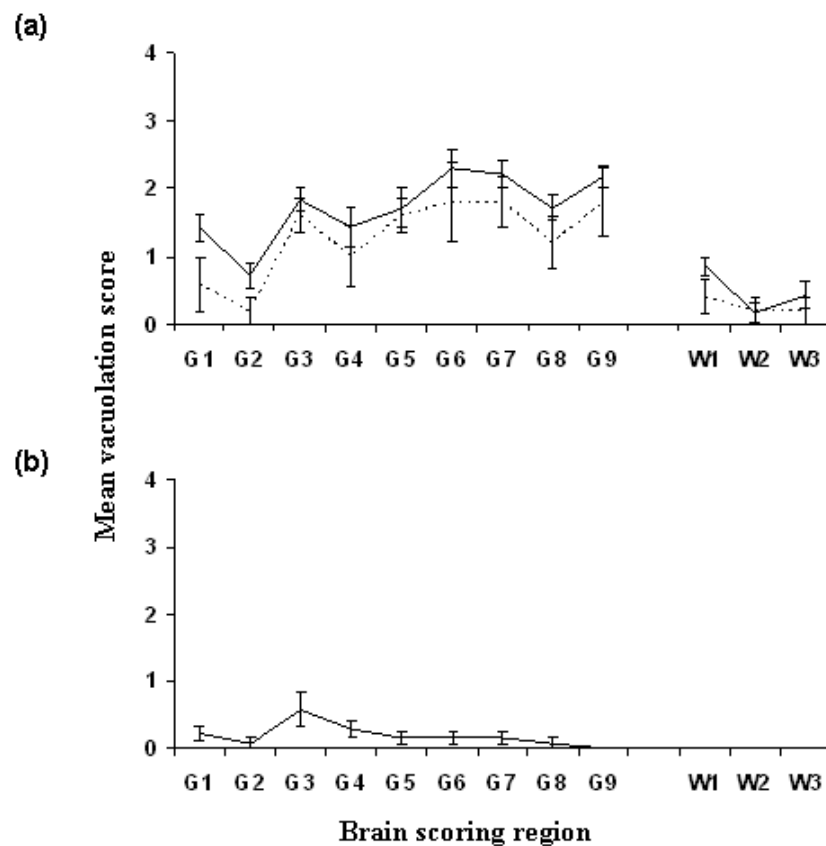
In mice inoculated with brain homogenate from two sCJD MM2 cases, only a single experimental group (RIII mice inoculated with CJD 12) produced sufficient mice with a positive score for TSE-specific vacuolation for the construction of a reliable lesion profile (Table 4.4). Furthermore, the development of clinical TSE disease in a proportion of RIII mice has allowed a lesion profile to be constructed from the seven mice displaying both clinical and pathological signs of a TSE (Figure 4.4a). However, it is difficult to draw any conclusions regarding the agent strain in sCJD when comparing lesion profiles constructed from mice at clinical end-point with those of mice sacrificed for other reasons. Therefore, a lesion profile was also constructed from the five RIII mice which had received a positive score for TSE-specific vacuolar pathology, but had a negative score for clinical signs of disease (Figure 4.4a). This allows a direct comparison between lesion profiles in RIII mice challenged with brain homogenate from the sCJD MM1, MV1 and MM2 subgroups. Patterns of vacuolar pathology were remarkably similar between RIII mice sacrificed at the clinical end-point of disease and those sacrificed with intercurrent illness or at their full life span (Figure 4.4a). Unsurprisingly, severity of vacuolation appeared higher in mice sacrificed with clinical TSE disease. Both lesion profiles showed subtle differences from those of RIII mice inoculated with sCJD MM1 (Figure 4.2) and MV1 brain homogenates (Figure 4.2), with a more widespread grey matter vacuolation involvement of the hippocampal region (G6) and the septum (G7). Although based on a single lesion profile, this may be indicative of subtle differences in the transmission properties of this sCJD MM2 case and other sCJD subgroups.

Differences were found in the clinical signs and in the proportion of mice showing vacuolar pathology between the two sCJD MM2 brain homogenates in the RIII mouse line. To determine whether these differences were reflected in the pattern of vacuolar degeneration in this mouse line, a lesion profile was constructed from the three RIII mice showing vacuolar degeneration following inoculation with brain homogenate from CJD 11 (Figure 4.4b). Although based on only three mice, pattern of vacuolar degeneration was similar to that observed with the MM1/MV1 brain isolates; however, the severity of vacuolar degeneration was lower. This is further

evidence that there are differences in the transmission properties of these two sCJD MM2 brain homogenates.

Figure 4.4: Lesion profiles for sCJD MM2A inoculated mice

(a) Lesion profile constructed from seven RIII mice with a positive score for clinical TSE disease and TSE-specific vacuolar pathology following challenge with brain homogenate from patient CJD 12 (solid line). Lesion profile constructed from five RIII mice with a positive score for TSE-specific vacuolar pathology with no signs of clinical disease following challenge with brain homogenate from patient CJD 12 (dotted line). (b) Lesion profile constructed from three RIII mice with a positive score for TSE-specific vacuolar pathology with no signs of clinical disease following challenge with brain homogenate from patient CJD 11. Lesion profiles are shown with standard error bars (\pm S.E.M).



4.3.3 Deposition of PrP in the mouse brain

Different TSE isolates often show different patterns of PrP deposition in the brain following transmission to mice of the same *Prn-p* genotype. When combined with incubation period data and lesion profile analysis, patterns of PrP deposition in the mouse brain can provide valuable information on agent strain (Bruce et al., 1989; Hecker et al., 1992; DeArmond et al., 1993). In these investigations, histological analysis of the mouse brain confirmed that all sCJD subgroups can transmit to wild-type mice, but with varying degrees of success. In the sCJD MM1, MV1 and MM2 subgroups, which transmit disease most successfully, not all inoculated mice showed TSE-associated vacuolar degeneration in the brain. Therefore, when selecting an immunohistochemical method for the detection of PrP in the brains of mice, the highly sensitive Catalyzed Signal Amplification System (CSA) was chosen in order to maximise the assay (Chapter 2, section 2.6.4.). Due to the similarity in the lesion profiles between different mouse lines, only two mouse lines were examined by immunohistochemistry; RIII from the *Prn-p^a* genotype and VM from the *Prn-p^b* genotype. Immunohistochemistry was carried out on 5µm formalin fixed, formic acid treated, brain sections from mice challenged with a minimum of two different brain homogenates from each sCJD subgroup. In each experiment, seven mice of each mouse line were analysed; five inoculated with CJD brain homogenate and two saline injected controls (where available). In the selection of CJD inoculated mice for immunohistochemical analysis, mice which had received a positive score for vacuolar pathology or if these were not available, mice which had the longest survival time were analysed. In addition, the C57BL mouse and C57BL x VM mouse which had shown evidence of vacuolar degeneration in the brain following inoculation with a sCJD VV2 (CJD 23) and VV1 (CJD 21) isolate were also analysed. Results for the immunohistochemical detection of PrP are shown in Table 4.7.

Table 4.7: Deposition of PrP in sCJD inoculated mice

Mouse line					
		CJD inoculated		Saline inoculated	
Source of brain inocula	Case	RIII	VM	RIII	VM
MM1	* C J D 1	5/5	5/5	0/2	0/2
	* C J D 7	4/5	4/5	0/2	0/2
	* C J D 8	5/5	3/5	0/2	0/2
	* C J D 9	5/5	4/5	0/2	0/2
MV1	*CJD 13	5/5	4/5	0/2	0/2
	*CJD 14	4/5	4/5	0/2	0/2
	*CJD 15	5/5	3/5	nd	nd
VV1	†CJD 20	0/5	0/5	0/2	0/2
	†CJD 21	0/6	0/6	0/2	0/2
MM2A	C J D 11	‡5/5	*4/4	nd	nd
	C J D 12	*2/5	§0/5	nd	nd
MV2A	†CJD 16	0/6	0/6	nt	nt
	†CJD 17	0/5	0/5	0/2	0/2
VV2A	†CJD 23	0/5	0/5	nt	nt
	†CJD 24	0/4	0/2	nt	0/2
	†CJD 25	0/5	0/5	0/2	0/2
	†CJD 26	0/5	0/5	0/1	0/2

* Groups of mice all scoring positive for TSE vacuolar degeneration

† Groups of mice all with a negative score for TSE vacuolar degeneration

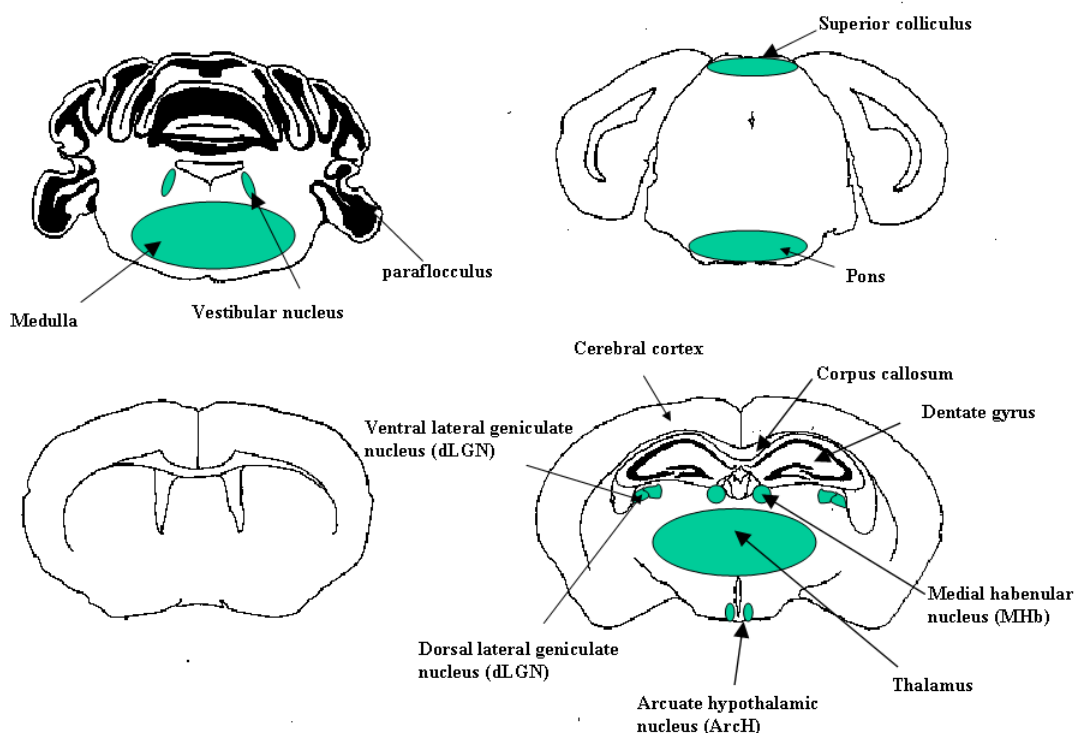
‡ 2 mice scored positive for PrP deposition in the absence of vacuolar degeneration

§ 2 mice scored positive for vacuolar degeneration

PrP deposition was observed in both the RIII and VM mice after inoculation of brain homogenate from the sCJD MM1, MV1 and MM2 cases, further confirming the successful transmission of a TSE from these subgroups. In contrast, PrP deposition was not detected in any mouse following challenge with brain homogenate from sCJD VV1, MV2 or VV2 cases. This includes the three mice which had shown a positive score for vacuolar pathology in the brain. The patterns of PrP accumulation in the sCJD MM1, MM2 and MV1 challenged mice are detailed below. Coronal sections of the mouse brain at the four levels analysed as a reference to the major anatomical regions of the brain associated with the deposition of PrP following challenge with sCJD subgroups are shown in Figure 4.5. All saline injected controls were negative for the presence of PrP.

Figure 4.5: Diagrammatic representation of the mouse brain

Diagrammatic representation of the four coronal levels used in the histological and immunohistological examination for TSE-related pathology in the mouse brain. Brain regions associated with the targeting of PrP pathology following experimental challenge with sCJD brain homogenate are highlighted in green and labelled.



4.3.3.1 sCJD MM1 subgroup

All mice analysed by immunohistochemistry following inoculation with sCJD MM1 brain homogenate had received a positive score for the presence of vacuolar degeneration in the brain. Immunohistochemistry showed PrP deposition in almost all mice tested (Table 4.7). Overall, PrP pathology was limited in the mice analysed. Faint granular deposits of PrP were consistently found targeting the dorsal lateral geniculate nucleus (dLGN), the medial habenular nucleus (MHb), the vestibular nucleus of the medulla and to a lesser extent the arcuate hypothalamic nucleus (ArchH) (Figure 4.6a -h). The deposition of PrP in the brains did not always correlate with the areas of most intense vacuolar pathology; however, PrP accumulation was observed in the superior colliculus and the cerebral cortex, regions of the brain associated with the most severe vacuolar pathology, in a few mice (Figure 4.7a and b). Amyloid plaques, a characteristic feature of certain sCJD subtypes, were observed in a single VM mouse analysed (Figure 4.7c and d). Although PrP pathology was limited in the mice, targeting of PrP accumulation was remarkably similar between the different mouse *Prn-p* genotypes and between mice inoculated with brain homogenate from different sCJD MM1 cases.

Figure 4.6: PrP deposition in sCJD MM1 inoculated mice

Fine granular deposits of PrP targeting the dorsal lateral geniculate nucleus (dLGN) in (a) RIII and (b) VM mice. Faint granular deposits of PrP within the medial habenular nucleus (MHb) in (c) RIII and (d) VM mice. Small focal deposits of PrP targeting the arcuate hypothalamic nucleus (ArchH) in (e) RIII and (f) VM mice. Granular accumulation of PrP accumulation in the vestibular nucleus of the medulla in (g) RIII and (h) VM mice. Sections shown were labelled with the monoclonal anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x200 (a, b, e, f, g, h), x400 (c, d).

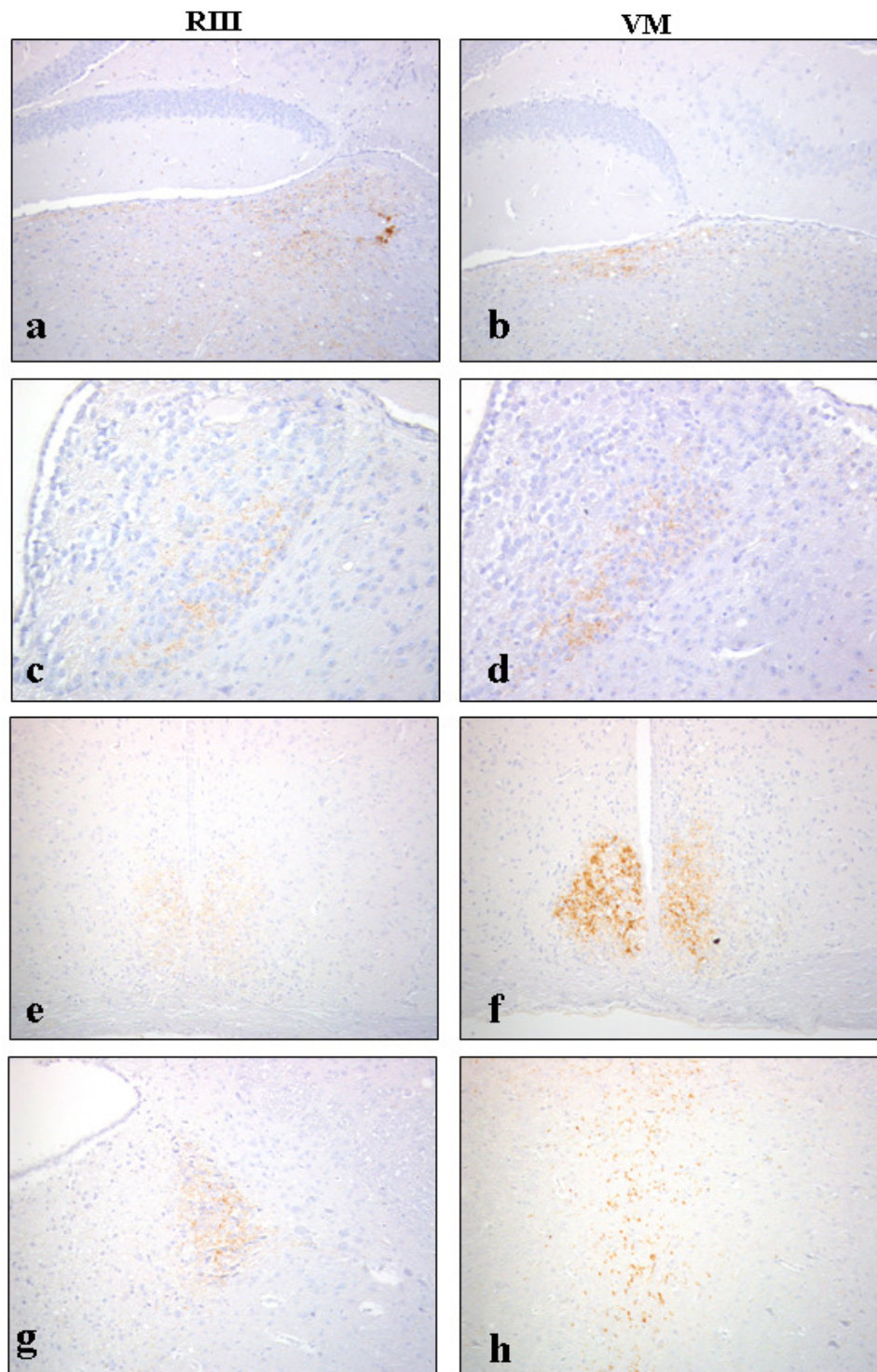
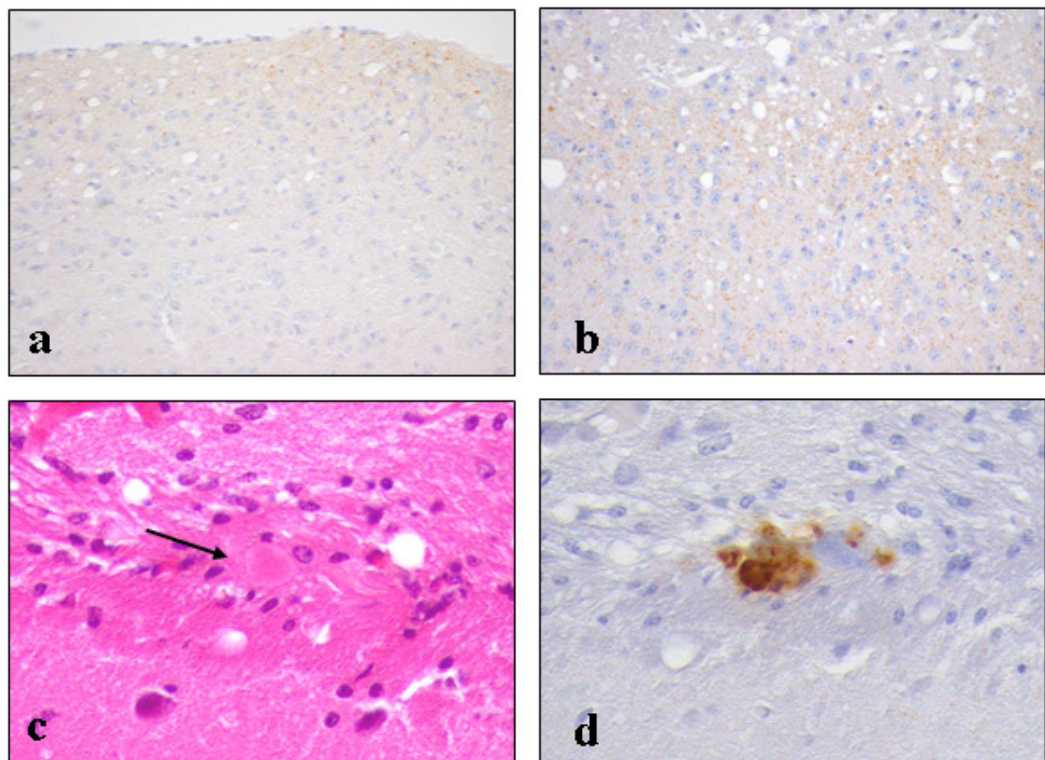


Figure 4.7: Further accumulations of PrP in sCJD MM1 inoculated mice

Fine granular deposits of PrP in **(a)** the superior colliculus and **(b)** the cerebral cortex in RIII mice. **(c)** Haematoxylin and eosin staining showing the presence of an amyloid plaque (arrow) within the corpus callosum of a VM mouse. **(d)** serial section from the same mouse immunolabelled for PrP. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x200 (a, b), x400 (c, d).



4.3.3.2 sCJD MV1 subgroup

All mice examined following challenge with MV1 brain homogenate had received a positive score for the presence of vacuolar degeneration in the brain. Immunohistochemical staining showed PrP deposition in the majority of mice tested (Table 4.7). The general targeting of PrP pathology was remarkably similar to that found in mice inoculated with sCJD MM1 brain homogenate, with localised deposits of faint granular PrP staining in the dLGN, MHb, ArcH and the vestibular nucleus of the medulla (Figure 4.8a-h). Occasional deposits of PrP were also observed in the superior colliculus, the cerebral cortex and the thalamus (Figure 4.9a-c). Staining patterns in the mouse brain were similar between the different mouse *Prn-p* genotypes (Figure 4.8) and between brain homogenate from different MV1 cases. Amyloid plaques were a common feature in the mice following challenge with sCJD MV1 brain isolates. This differs from the pattern in the MM1 transmissions where amyloid plaques were only observed in the VM mice (Figure 4.7d). Although observed in both the RIII and VM mice, these plaques were a more prominent feature in the VM mouse line.

Figure 4.8: PrP deposition in sCJD MV1 inoculated mice

Granular deposits of PrP targeting the dorsal lateral geniculate nucleus (dLGN) in (a) RIII and (b) VM mice. Faint granular deposits of PrP within the medial habenular nucleus (MHb) in (c) RIII and (d) VM mice. Small focal deposits of PrP targeting the arcuate hypothalamic nucleus (Arch) in (e) RIII and (f) VM mice. Granular deposition of PrP in the reticular nucleus of the medulla in (g) RIII and (h) VM mice. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x100(c, d), x200 (a, e, f, g, h), x400 (b).

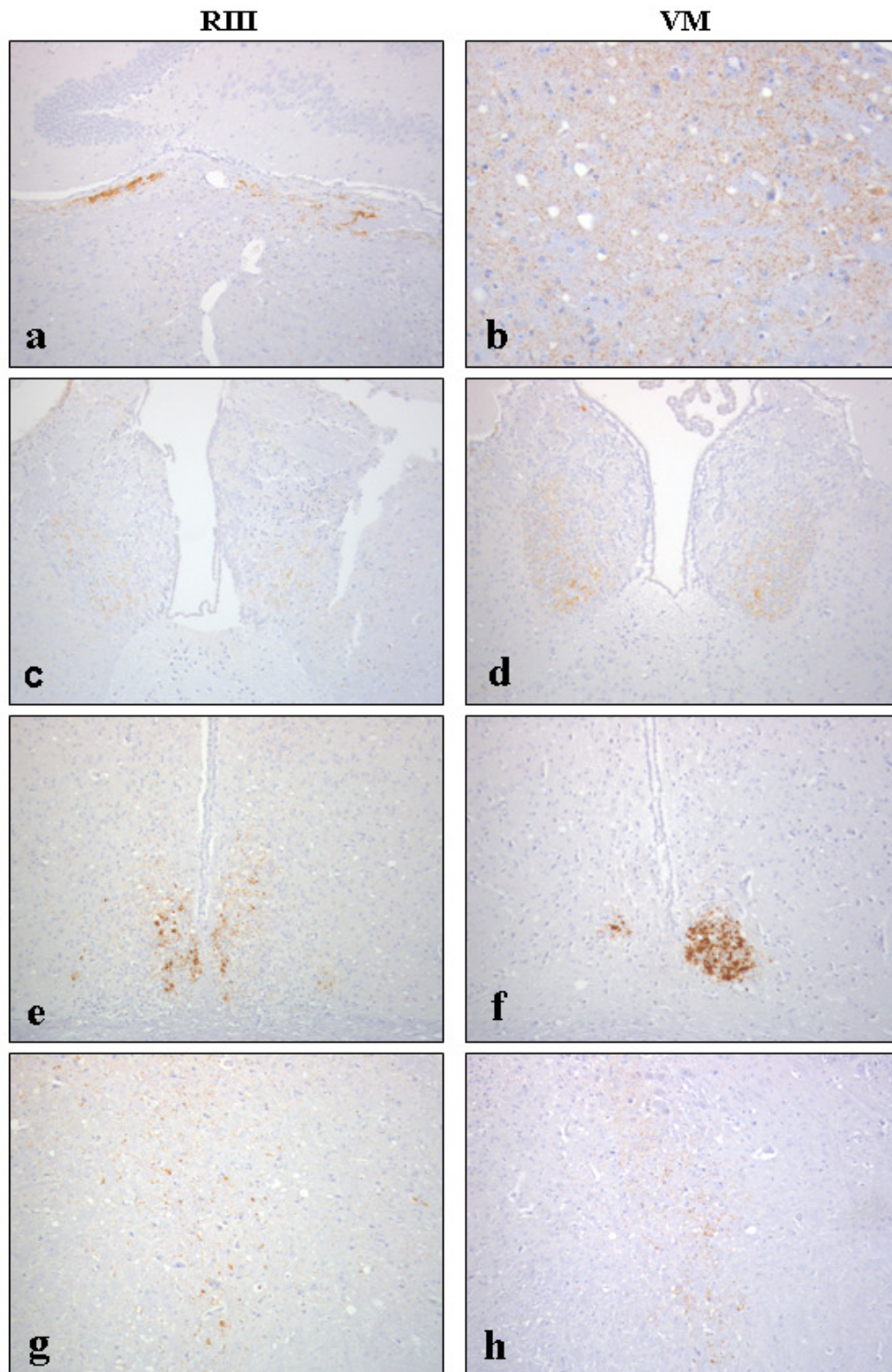
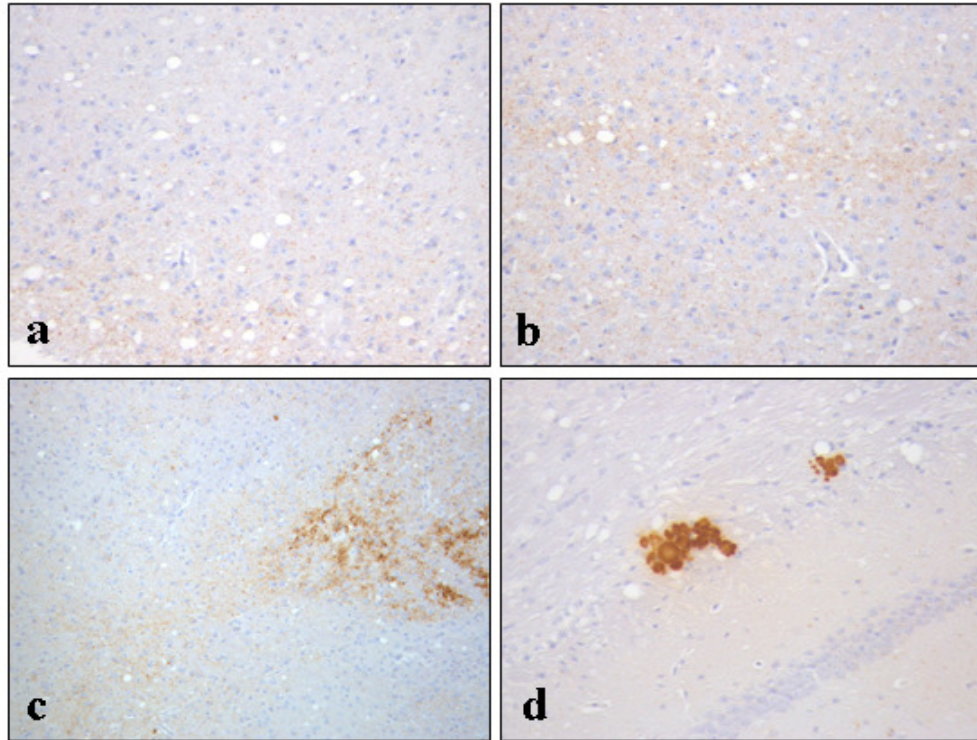


Figure 4.9: Further accumulations of PrP in sCJD MV1 inoculated mice

Fine granular deposits of PrP throughout the **(a)** superior colliculus and **(b)** cerebral cortex in RIII mice. **(c)** Granular deposition of PrP throughout the thalamus in a VM mouse. **(d)** Section of a VM mouse brain immunolabelled for PrP showing the presence of amyloid plaques within the corpus callosum. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x100 (c), x200 (a, b), x400 (d).

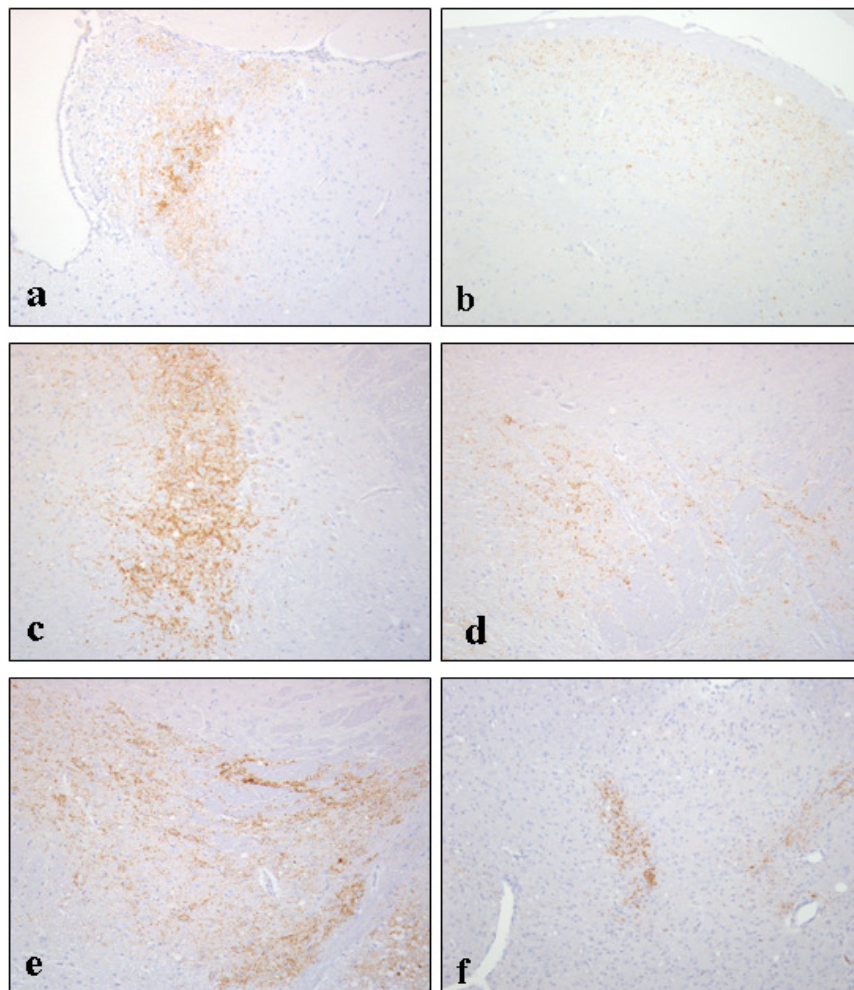


4.3.3.3 sCJD MM2A subgroup

Consistent with other strain typing parameters, subtle differences were found in the deposition of PrP between the two sCJD MM2 cases under investigation. Immunohistochemistry for the prion protein showed the deposition of PrP in almost all mice inoculated with brain homogenate from CJD 11, even in two RIII mice which showed no evidence of vacuolar pathology. In contrast, only two RIII mice showed evidence of PrP accumulation in the brain after challenge with brain homogenate from CJD 12 (Table 4.7). Mice challenged with brain homogenate from CJD 11, showed a widespread distribution of small granular deposits of PrP in brain regions (vestibular nucleus of the medulla, MHb and dLGN) consistently targeted following challenge with brain homogenate from sCJD MM1 and MV1 subgroups (Figure 4.10 a-c). In addition, peri-neuronal deposits of PrP were consistently found targeting the pons in the RIII mice, a region of the brain not commonly associated with mice challenged with sCJD MM1 and MV1 brain homogenates (Figure 5.10d). In the two mice showing PrP pathology following challenge with CJD 12, small intense granular deposits of PrP were restricted to the pons and the thalamus (Figure 4.10 g and h). Amyloid plaques were not a feature in these mice.

Figure 4.10: PrP deposition in sCJD MM2A inoculated mice

Immunohistochemical labelling of the abnormal prion protein in mice following inoculation of brain homogenate from CJD 11 (a, b, c, d) and CJD 12 (e and f). Granular deposition of PrP in the (a) medial habenular nucleus and (b) dorsal lateral geniculate nucleus of an RIII mouse. (c) Intense focal deposits of PrP targeting the vestibular nucleus of the medulla in a VM mouse. Peri-neuronal and granular deposition of PrP in the pons of an RIII mouse after challenge with brain homogenate from (d) CJD 11 and (e) CJD 12. (f) Small intense deposits of PrP located in the thalamus of an RIII mouse. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification x200.



4.3.4 Biochemical isotyping of PK resistant PrP^{Sc}

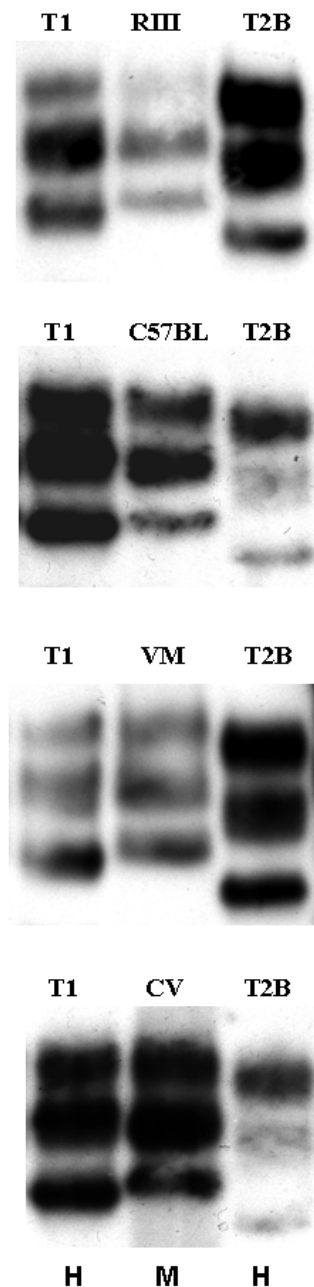
Characterisation of the PrP^{res} type in the mouse brain following inoculation with brain homogenate from all sCJD subgroups was carried out using a standard Western blot protocol used in the diagnostic typing of human TSEs (Chapter 2, section 2.7). Western blotting was carried out on extracts of frozen CNS tissue from mice ($n \geq 2$) of all *Prn-p* genotypes inoculated with brain homogenate from a minimum of two sCJD cases from each sCJD subgroup. Mice preferentially selected for analysis had received a positive score for vacuolar pathology in the brain. In the absence of any mice with a positive vacuolar score, mice with the longest survival time were selected. Western blot analysis was carried out on 5µl extracts of a 10% brain tissue weight to buffer volume (w/v) in mice that had received a positive score for vacuolar degeneration unless otherwise stated. In mice showing no evidence of vacuolar pathology, Western blotting was carried out on 100µl of a 10% w/v extract concentrated by centrifugation prior to analysis. Mouse extracts were run alongside a sCJD MM1 and a vCJD MM2B diagnostic brain sample as reference controls for both fragment mobility and glycoform ratio.

4.3.4.1 sCJD MM1 subgroup

All mice analysed had evidence of vacuolar degeneration in the brain. PrP^{res} was readily detected in CNS extracts of all mice analysed, further evidence for the successful transmission of disease from MM1 brain homogenate. PrP^{res} in mouse brain extracts showed a glycosylation pattern closely resembling that of the sCJD MM1 diagnostic brain extract (Figure 4.11). A subtle difference in the electrophoretic mobility of the unglycosylated form of the protein was found between the mouse and human brain samples. This suggests some variation in the size of the protease-resistant core of the mouse and human protein, either because of differences in the exact truncation point for PK or because of sequence differences between human and murine PrP. PrP^{res} type was similar between mice of different *Prn-p* genotypes (Figure 4.11) and between mice of the same *Prn-p* genotype after inoculation with brain homogenate from different sCJD MM1 cases. These results indicate that the biochemical phenotype of sCJD in MM1 individuals is maintained on primary transmission to wild-type mice.

Figure 4.11: Western blot analysis of PrP^{res} in sCJD MM1 inoculated mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after experimental transmission of sCJD MM1 brain homogenate. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).

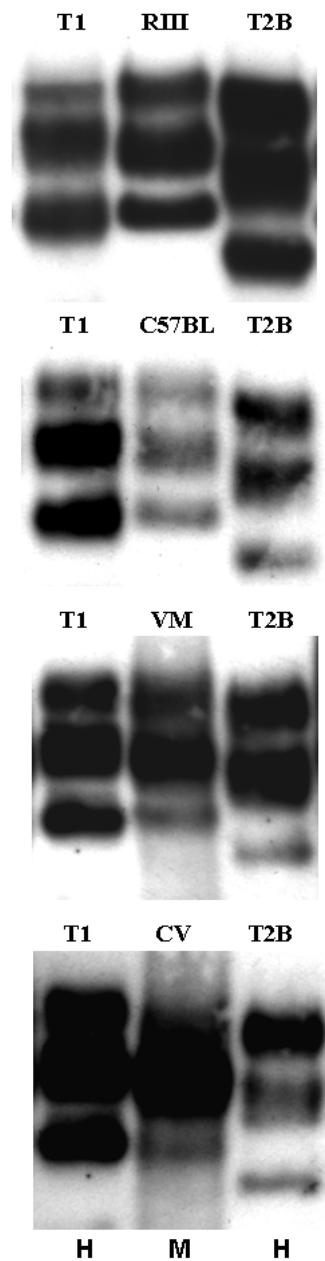


4.3.4.2 sCJD MV1 subgroup

All mice analysed for the presence of PrP^{res} in CNS tissue extracts had received a positive score for TSE-specific vacuolar degeneration in the brain. As seen in sCJD MM1 inoculated mice, PrP^{res} was readily detected in all brain extracts analysed following experimental challenge with MV1 brain homogenate. This further confirms transmission of a TSE to mice from sCJD MV1 brain homogenate. The glycosylation pattern and electrophoretic mobility of the recipient mouse PrP^{res} was consistent with those of the sCJD MM1 diagnostic standard (Figure 4.12) and the PrP^{res} type 1 detected in the sCJD MV brain homogenate used in the inoculation of the mice (Figure 3.2). Furthermore, the PrP^{res} type detected in the brains of the MV1 inoculated mice was closely similar to that observed in MM1 inoculated mice (Figure 4.11). Again, a similar PrP^{res} type was observed in mice from the different *Prn-p* genotypes (Figure 4.12) and between different MV1 brain homogenates. These results confirm that the biochemical phenotype of MV1 sCJD cases is conserved following primary transmission to wild-type mice.

Figure 4.12: Western blot analysis of PrP^{res} in sCJD MV1 inoculated mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after experimental transmission of sCJD MV1 brain homogenate. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).



4.3.4.3 sCJD VV1 subgroup

Only one mouse (a C57BL x VM mouse) showed vacuolar evidence of a TSE following experimental challenge with brain homogenate from a sCJD VV1 patient (Table 4.3). Western blot analysis of a 10µl CNS extract from this mouse further confirmed the diagnosis of a TSE with the detection of PrP^{res}. The resulting PrP^{res} banding pattern had a glycosylation pattern and molecular weight of the unglycosylated fragment closely resembling that of the type 1 sCJD MM diagnostic standard (Figure 4.13) and the type 1 sCJD VV brain homogenate used in the inoculation of the mouse (Figure 3.2). Western blotting on a 100µl CNS extract from mice of all *Prn-p* genotypes which had shown no pathological evidence of a TSE, also showed no detectable levels of PrP^{res} in the mouse brain (Figure 4.14). Western blotting on these CNS extracts without proteinase K treatment, confirmed the presence of PrP in the mouse brains (Figure 4.15). Although based on a single mouse showing detectable levels of PrP^{res} in the brain, this result suggests that in mice which do show positive transmission from sCJD VV1 cases, the biochemical phenotype of the donor tissue is maintained. Furthermore, these results confirm that of previous strain typing parameters which indicate that transmission of sCJD from VV1 cases does occur but is rare.

Figure 4.13: Detection of PrP^{res} in a sCJD VV1 inoculated mouse

Western blot analysis of PrP^{res} in a 10µl CNS extract from a C57BL x VM (CV) mouse which had shown positive TSE vacuolar pathology after inoculation with a sCJD VV1 (CJD 21) brain homogenate. Tissues are analysed after digestion with proteinase K. Mouse sample (M) is run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).

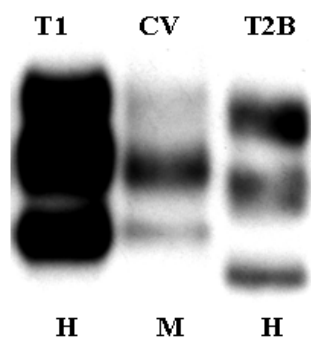


Figure 4.14: Western blot analysis of PrP^{res} in sCJD VV1 inoculated mice

Western blot analysis of PrP^{res} in 100µl centrifugal concentrated CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after experimental transmission of sCJD VV1 brain homogenate. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).

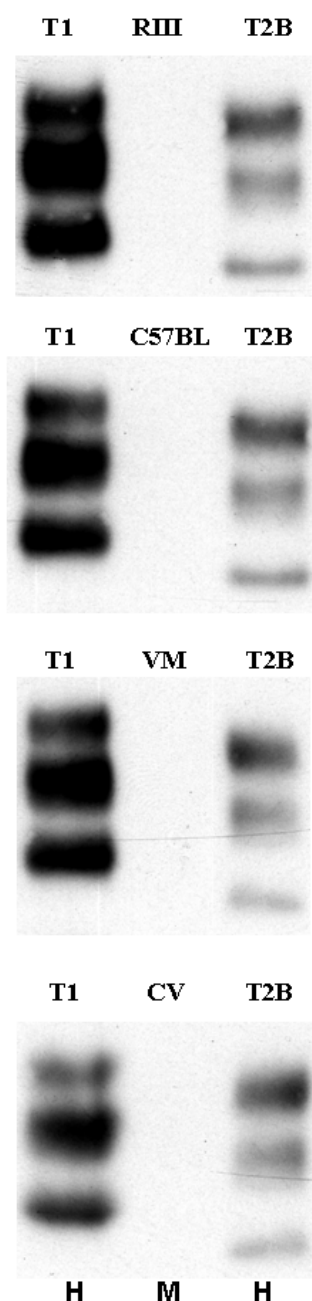
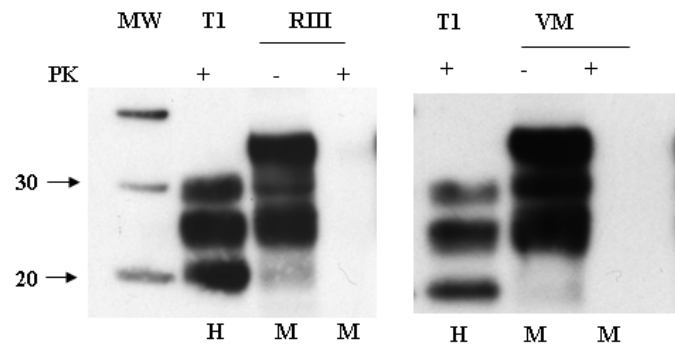


Figure 4.15: Confirmation of PrP in sCJD VV1 inoculated mice

Western blot analysis of a *Prn-p^a* (RIII) and *Prn-p^b* (VM) mouse CNS extract analysed with (+) and without (-) proteinase K treatment. Mouse samples (M) were run alongside a human (H) sCJD MM1 type 1 (T1) diagnostic standard. Molecular weight markers are shown in kDa.

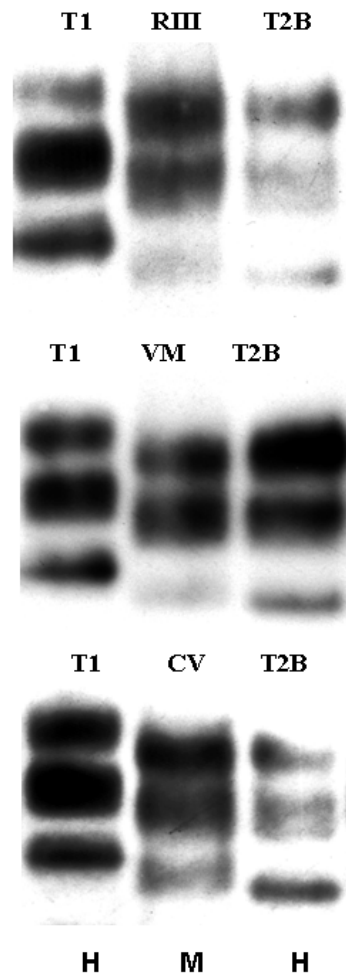


4.3.4.4 sCJD MM2A subgroup

Frozen mouse CNS tissue for Western blot analyses was only available from a single sCJD MM2 experiment (CJD 12). All mice investigated from this experiment had received a positive score for TSE-specific vacuolar pathology in the brain. PrP^{res} was readily detected in all RIII, VM and C57BL x VM CNS extracts examined. C57BL mice were not challenged with this brain homogenate and were therefore not available for testing. The PrP^{res} banding pattern in CNS extracts of mice of all *Prn-p* genotypes (the two homozygotes and the heterozygote F₁ cross) showed a molecular weight of the unglycosylated fragment of approximately 19kDa, similar to that seen in the vCJD diagnostic standard (Figure 4.16). However, the glycosylation pattern more closely resembled that of the sCJD MM2 brain homogenate used in the inoculations (Figure 3.2). Crucially, this banding pattern differed from sCJD MM1 diagnostic standard in terms of the molecular weight of the unglycosylated fragment (Figure 4.16). These results further confirm the successful transmission of a TSE from sCJD MM2 cases and show that the biochemical phenotype of this MM2 sCJD brain homogenate was maintained on transmission to mice. Furthermore, the detection of PrP^{res} in the VM challenged mice suggests that Western blot analysis is a more sensitive assay in the detection of PrP in the sCJD inoculated mice when compared to immunohistochemistry, as immunohistochemistry showed no evidence of PrP in the brains of VM inoculated mice.

Figure 4.16: Western blot analysis of PrP^{res} in sCJD MM2 inoculated mice

Western blot analysis of PrP^{res} in CNS samples of RIII, VM and C57BL x VM (CV) mice after experimental transmission of sCJD MM2 brain homogenate. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).



4.3.4.5 sCJD MV2A subgroup

Western blot analyses of frozen mouse CNS tissue after primary challenge with brain homogenate from sCJD MV2 cases showed detectable levels of PrP^{res} in a single RIII mouse examined. This same RIII mouse had also provided the only positive score for the presence of TSE-specific vacuolar pathology in the brain following histological examination. Surprisingly, the PrP^{res} type of this RIII mouse had an unglycosylated fragment with a molecular weight of approximately 21kDa. This molecular weight closely resembles that of the sCJD MM1 diagnostic standard and differs from that of the vCJD type 2B diagnostic standard (Figure 4.17) and that of the type 2 PrP^{res} detected in the inoculum (Figure 3.2).. All other extracts from RIII, C57BL, VM and the C57BL x VM mice showed no evidence of PrP^{res} in the brain, even after analysis of 100µl concentrated samples (Figure 4.18). However, PrP was confirmed in these mouse CNS extracts in the absence of PK digestion (Figure 4.19) This result supports observations made from the previous strain typing parameters, that transmission of a TSE from sCJD MV2 cases is rare. However, the PrP^{res} type detected in the brain of the one RIII mouse suggests that the biochemical phenotype of the donor MV2 sCJD case was not maintained on primary transmission but resulted in a PrP^{res} type more reminiscent of that detected in a sCJD MM1 and MV1 inoculated mouse. It is interesting that the vacuolar pathology of this single mouse also resembled that seen in sCJD MM1 challenged RIII mice.

Figure 4.17: Detection of PrP^{res} in a sCJD MV2 inoculated mouse

Western blot analysis of PrP^{res} in a 10µl CNS extract from an RIII mouse which had shown positive TSE vacuolar pathology after inoculation with a sCJD MV2 (CJD 16) brain homogenate. Tissues are analysed after digestion with proteinase K. Mouse sample (M) is run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).

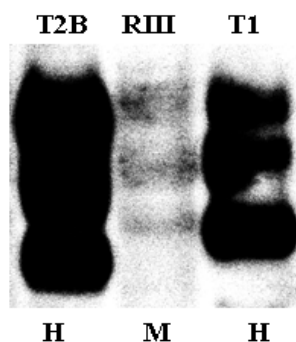


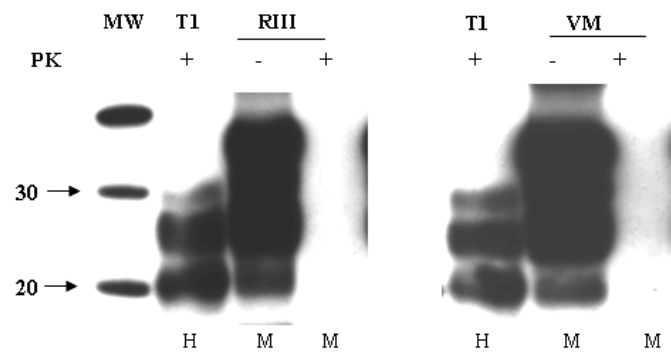
Figure 4.18: Western blot analysis of PrP^{res} in sCJD MV2 inoculated mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after experimental transmission of sCJD MV2 brain homogenate. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).



Figure 4.19: Confirmation of PrP in sCJD MV2 inoculated mice

Western blot analysis of a *Prn-p^a* (RIII) and *Prn-p^b* (VM) mouse CNS extract analysed with (+) and without (-) proteinase K treatment. Mouse samples (M) were run alongside a human (H) sCJD MM1 type 1 (T1) diagnostic standard. Molecular weight markers are shown in kDa.



4.3.4.6 sCJD VV2A subgroup

PrP^{res} was detected after experimental challenge with sCJD from a single VV2 source (CJD 23) but only in one C57BL mouse. This same C57BL mouse had also received a positive score for vacuolar degeneration. The PrP^{res} type in this mouse showed a mobility of the unglycosylated fragment resembling that of the vCJD diagnostic standard (Figure 4.20) and consistent with that detected in the sCJD VV2 inoculum used in the transmission (Figure 3.2). A feature of the experimentally infected mouse that was not observed in the human vCJD MM2B standard or the inoculum was the appearance of the unglycosylated fragment as a doublet composed of a distinct upper and lower band (Figure 4.20). All other mice tested showed no evidence of PrP^{res} in the brain, even after analysis of 100µl concentrated samples (Figure 4.21). Again, Western blot analysis of these mouse CNS samples without any PK digestion had confirmed the presence of PrP (Figure 4.22). This result confirms the previous observations from the other strain typing parameters that transmission from sCJD VV2 cases does occur but is rare. In addition, this result also indicates that the biochemical phenotype of sCJD VV2 cases showing positive transmission was conserved following on transmission to wild-type mice in these experiments.

A representative Western blot comparing PrP^{res} type in recipient mice after primary challenge with brain homogenate from sCJD MM1, MV1 and MM2 cases is shown in Figure 4. 23

Figure 4.20: Detection of PrP^{res} in a sCJD VV2 inoculated mouse

Western blot analysis of PrP^{res} in a 40µl CNS extract from a C57BL mouse which had shown positive TSE vacuolar pathology after inoculation with a sCJD VV2 (CJD 23) brain homogenate. Tissues are analysed after digestion with proteinase K. Mouse sample (M) is run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B). (U) Upper band of the unglycosylated fragment, (L) Lower band of the unglycosylated fragment.

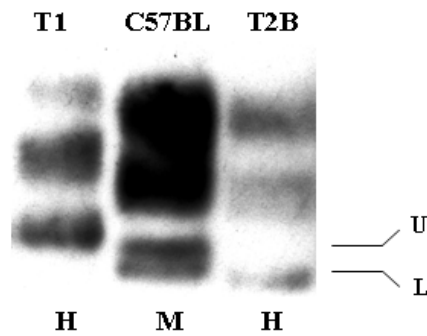


Figure 4.21: Western blot analysis of PrP^{res} in sCJD VV2 inoculated mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after experimental transmission of sCJD VV2 brain homogenate. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).

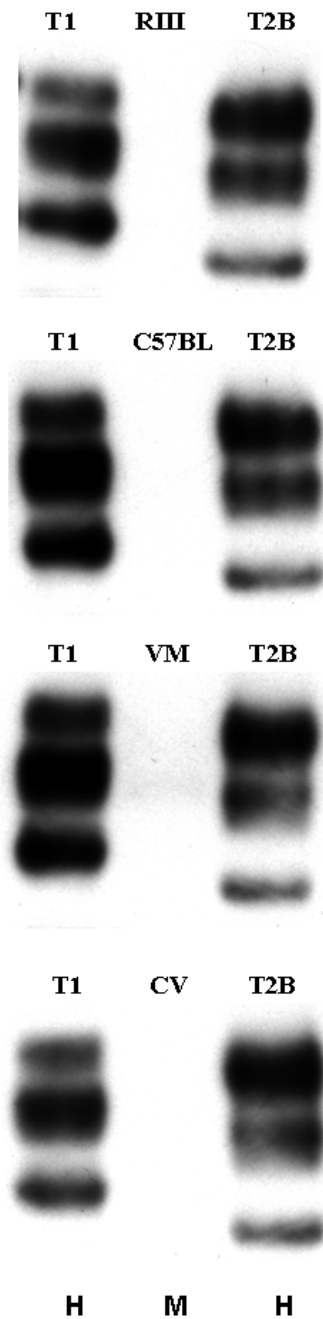


Figure 4.22: Confirmation of PrP in sCJD VV2 inoculated mice

Western blot analysis of a *Prn-p^a* (RIII) and *Prn-p^b* (VM) mouse CNS extract analysed with (+) and without (-) proteinase K treatment. Mouse samples (M) were run alongside a human (H) sCJD MM1 type 1 (T1) diagnostic standard. Molecular weight markers are shown in kDa.

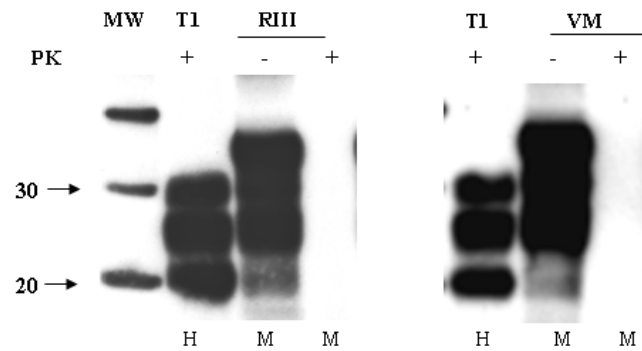
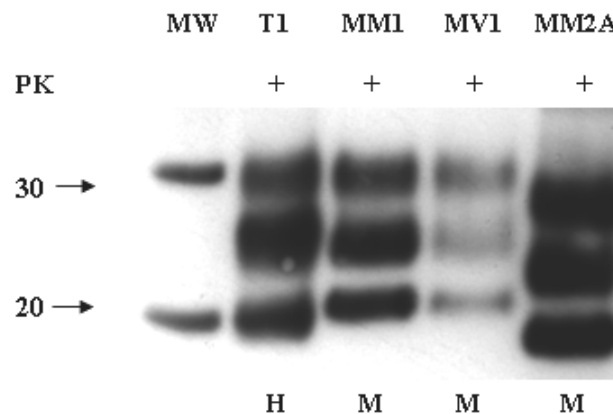


Figure 4.23: PrP^{res} in RIII mice after challenge with different sCJD subgroups

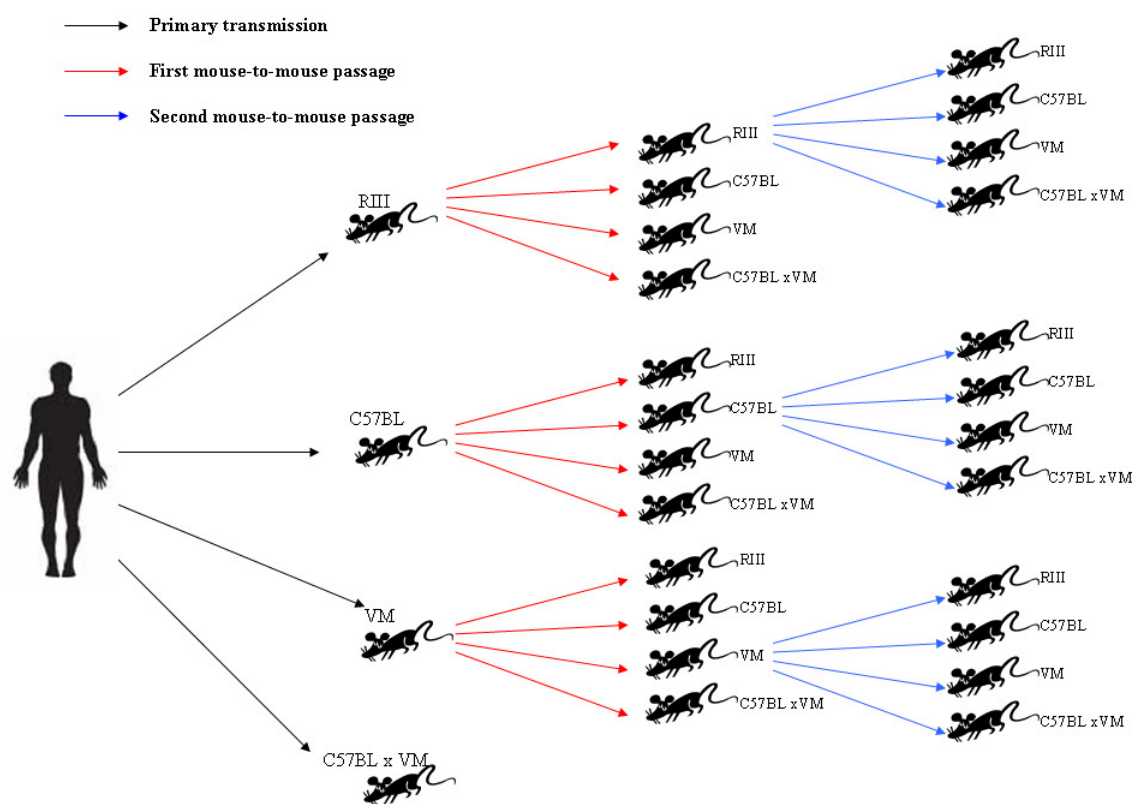
All mouse CNS extracts are analysed after (+) digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) diagnostic standard. Molecular weight makers (MW) are shown in kDa



4.4 Experimental results: mouse-to-mouse passage

Different TSE strains are often isolated from a single source by serial passage through mice of different *Prn-p* genotypes, so a full characterisation of a source includes strain typing following primary transmission to mice and on subsequent mouse-to-mouse transmission of the TSE isolate. In this thesis, further characterisation of the agent strain in sCJD was carried out by conducting two serial mouse-to-mouse passages of brain isolates derived from two different sCJD MM1 frontal cortex samples (CJD 1 and CJD 5) and a single sCJD cerebellar sample (CJD 2). The sCJD MM1 subtype was selected for further sub-passage because primary transmissions indicated that this sCJD subgroup transmits most successfully in the wild-type mouse panel, with the largest numbers of mice having had a positive score for the presence of TSE-specific vacuolar pathology (Figure 4.1). In addition, methionine homozygosity at *PRNP* codon 129 is a recognised risk factor in the development of sCJD with the presence of type 1 PrP^{res} in the brain comprising the largest proportion of sCJD cases. Brain homogenates were prepared as previously described (Chapter 2, section 2.3.4.) from the brain of a single RIII, VM and C57BL mouse showing TSE-specific vacuolation in the brain after primary challenge with sCJD MM1 brain homogenate. Groups of approximately 12 mice from each mouse *Prn-p* genotype were inoculated via a single IC injection. As in the primary transmission of sCJD into wild-type mice, only mice surviving over 250dpi were included in the analysis. Saline inoculated controls were not carried out in the sub-passage experiments. Figure 4.24 shows a diagrammatic representation of the sub-passages carried out in this thesis.

Figure 4.24: Diagrammatic representation of sub-passage experiments



4.4.1 Incubation periods and incidence of disease

Each sCJD brain isolate produced similar results in all mouse lines challenged, regardless of the brain region used in the original primary transmission. The incidence of clinical and vacuolar pathology in mice after serial passage of sCJD brain isolates in *Prn-p^a* and *Prn-p^b* mice are detailed in Table 4.8a and Table 4.8b respectively.

The numbers of mice sacrificed as a result of clinical TSE disease was more frequent than was observed on primary transmission (Table 4.1), a feature indicative of a removal of the ‘species barrier’ which was present during the original human to mouse transmission. The most striking difference was found in the VM mouse line in which almost 100% of mice challenged with brain isolates passaged through *Prn-p^b* mice developed clinical signs of disease with TSE-specific vacuolar pathology confirmed at post-mortem (Table 4.8b). In contrast, only a small proportion of VM mice challenged with brain isolates passaged through *Prn-p^a* (RIII and C57BL) mice resulted in clinical signs of a TSE (Table 4.8a). In the RIII-passage of brain isolates from CJD 2, no clinical or histological evidence of a TSE disease was found in any of the mice inoculated (Table 4.8a). This was not the case in the RIII-passage of brain isolates from CJD 1 or CJD 5. Although titres of infectivity in the inoculum are not known, these contrasting results may reflect low levels of infectivity within the RIII mouse brain selected from the primary transmission of CJD 2 for further passage in mice. In support of this proposal, immunohistochemical analysis failed to show any evidence of PrP in the RIII mouse selected as inocula for the subsequent mouse passage. A further explanation is that infectivity levels may be lower in sCJD cerebellar tissue compared to frontal cortex. However, results from the primary transmission of sCJD MM1 brain isolates show no evidence of this regional difference in levels of infectivity.

Table 4.8a: Serial passage of sCJD MM1 brain isolates in *Prn-p^a* mice

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice after two serial mouse-to-mouse passages of isolates derived from brain homogenates from three sCJD MM1 cases. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Incubation periods in days are shown in brackets (mean \pm S.E.M. for groups with >5 clinical cases. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Recipient mouse lines							
	RIII		C57BL		VM		C57BL x VM	
Donor tissue source	Clin	Vac path	Clin	Vac path	Clin	Vac path	Clin	Vac path
*CJD 1 RIII 1 st pass	3/10 (820, 834, 944)	4/10	3/8 (939, path -ve, path -ve)	1/8	3/10 (562, 649, 734)	9/10	0/10	7/10
2 nd pass	2/11 (627, 1005)	4/11	0/4	0/4	0/9	5/9	0/9	4/9
C57BL 1 st pass	3/12 (780, 806, 862)	8/12	2/9 (885, path -ve)	6/9	2/7 (776, 875)	7/7	0/10	6/10
2 nd pass	1/11 (715)	5/11	2/7 (894, path -ve)	3/7	0/11	9/11	0/10	8/10
†CJD 2 RIII 1 st pass	1/9 (path -ve)	0/9	0/7	0/7	2/8 (path -ve)	0/8	0/8	0/8
C57BL 1 st pass	5/15 (898 \pm 28)	9/15	1/12 (path -ve)	7/12	1/11 (820)	11/11	0/9	6/9
2 nd pass	0/11	7/11	1/9 (path -ve)	5/9	1/8 (path -ve)	5/8	0/10	9/10
*CJD 5 RIII 1 st pass	5/10 (847 \pm 17)	8/10	4/10 (485, 698, 835, path -ve)	5/10	3/11 (625, 716, path -ve)	8/11	0/10	6/10
2 nd pass	2/11 (855, 889)	9/11	1/7 (887)	4/7	2/10 (701, 764)	10/10	0/7	7/7
C57BL 1 st pass	4/10 (521, 797, 883, path -ve)	6/10	2/12 (856, 883)	6/12	1/12 (751)	12/12	1/12 (881)	11/12
2 nd pass	1/7 (path -ve)	0/7	1/10 (path -ve)	2/10	0/9	5/9	0/8	2/8

* frontal cortex isolates † cerebellar cortex isolates

Table 4.8b: Serial passage of sCJD MM1 brain isolates in *Prn-p^b* mice

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice after two serial mouse-to-mouse passages of isolates derived from brain homogenates from three sCJD MM1 cases. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Incubation periods in days are shown in brackets (mean \pm S.E.M. for groups with >5 clinical cases). In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Recipient mouse line							
	RIII		C57BL		VM		C57BL x VM	
Donor tissue source	Clin	Vac path	Clin	Vac path	Clin	Vac path	Clin	Vac path
*CJD 1 VM 1 st pass	6/12 (856 \pm 36)	6/12	4/9 (729, 764, 868, path –ve)	6/9	9/11 (563 \pm 8)	11/11	0/9	7/9
2 nd pass	2/10 (864, path –ve)	7/10	1/7 (path –ve)	3/7	11/11 (542 \pm 8)	11/11	0/8	6/8
†CJD 2 VM 1 st pass	6/16 (791 \pm 12)	7/16	3/9 (776, 820, 844)	7/9	11/11 (548 \pm 9)	11/11	1/7 (545)	6/7
2 nd pass	1/11 (path –ve)	6/11	0/6	5/6	11/12 (493 \pm 2)	12/12	0/10	10/10
*CJD 5 VM 1 st pass	3/12 (804, 962, 962)	7/12	6/9 (915 \pm 27)	7/9	10/11 (558 \pm 6)	11/11	0/10	9/10
2 nd pass	0/10	10/10	0/7	3/7	9/10 (514 \pm 6)	10/10	0/8	8/8

* frontal cortex isolates

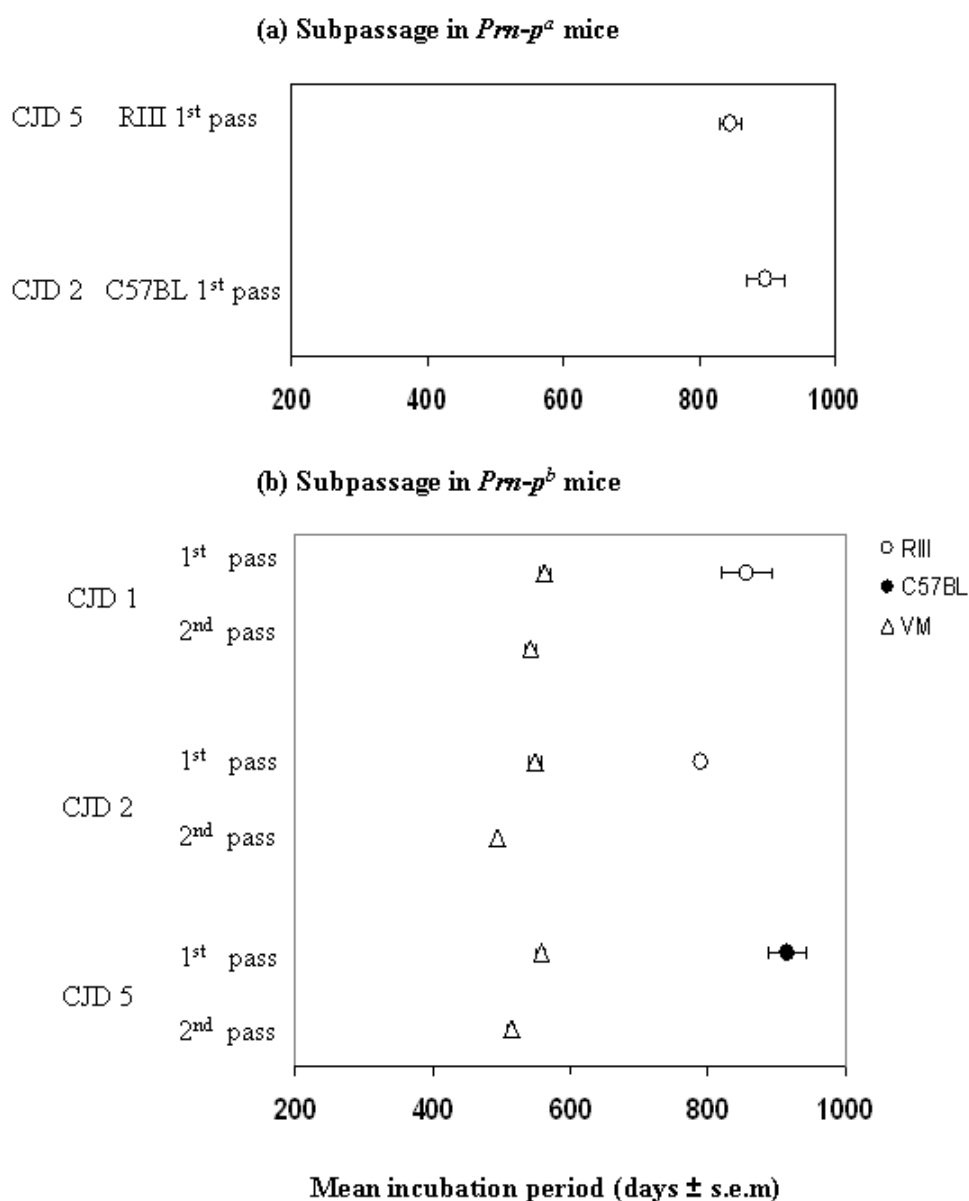
† cerebellar cortex isolates

The development of a progressive clinical disease in a proportion of the inoculated mice has provided mean incubation period data in some experimental groups. Mean incubation periods were drawn from groups with a minimum of five mice that had recieved a positive score for both clinical signs of disease and for the presence of TSE-specific vacuolation in the brain. Incubation periods are shown in Figure 4.25. All VM mice challenged with isolates passaged through *Prn-p^b* (VM) mice developed disease with mean incubation periods up to a standard clinical endpoint of 563 ± 8 , 558 ± 6 and 548 ± 9 days (\pm S.E.M) on first mouse-to-mouse passage with a slight shortening on a further passage (Table 4.8b). In contrast, incubation periods were not observed in any VM group after passage in *Prn-p^a* mice. This suggests the isolation of two distinct mouse-passaged strains of agent recognised in VM mice by passage in the different mouse *Prn-p* genotypes. Incubation period data was not a common feature of the other mouse lines after passage in either mouse *Prn-p* genotype. Interestingly, with the exception of the VM mice injected with VM-passaged isolates, groups with incubation period data on first mouse-to-mouse passage, failed to show an incubation period on further passage. This is in contrast to the sub-passage of other TSE isolates where incubation periods generally shorten after further mouse-to-mouse passages.

As observed in the primary transmission of sCJD MM1 brain homogenate, the failure to show clinical signs of disease does not indicate a failure to transmit disease, as vacuolar degeneration typical of TSE infection was seen in the brains of a proportion of the mice dying with intercurrent disease in almost all experimental groups (Table 4.8a and b).

Figure 4.25: Incubation times in mice after sub-passage of sCJD MM1

Mean incubation periods in RIII, C57BL and VM mice after first and second mouse passage of sCJD MM1 brain isolates through (a) *Prn-p^a* and (b) *Prn-p^b* mice. Data are mean \pm S.E.M



4.4.2 Patterns of vacuolar pathology- lesion profiles

In the primary transmission of sCJD brain homogenates, the lack of any clinical TSE disease in inoculated mice meant that lesion profiling was predominantly carried out using mice showing only positive vacuolar pathology. Profiles from the primary challenge of sCJD MM2 brain homogenate in RIII mice indicate that patterns of vacuolar pathology are closely similar between mice dying with clinical TSE disease and those dying with intercurrent illness or at full life span, although unsurprisingly the pathology is less severe in non-clinical mice (Figure 4.4). In the sub-passage of sCJD brain isolates, numbers of mice showing both clinical and vacuolar pathology were low in most groups. Therefore, in the construction of lesion profiles after sub-passage of sCJD, mice that had received a positive score for TSE specific vacuolation and which were sacrificed with either intercurrent disease or at clinical end-point of disease were included in the same lesion profile. Vacuolar degeneration was seen in all four mouse lines injected with sCJD MM1 brain homogenates passaged in the different mouse *Prn-p* genotypes; however, not all experimental groups had sufficient mice (≥ 5 mice) with a positive score for TSE-specific vacuolation for the construction of a lesion profile. Lesion profiles were remarkably similar for the three sCJD MM1 brain isolates and are shown in Figure 4.26.

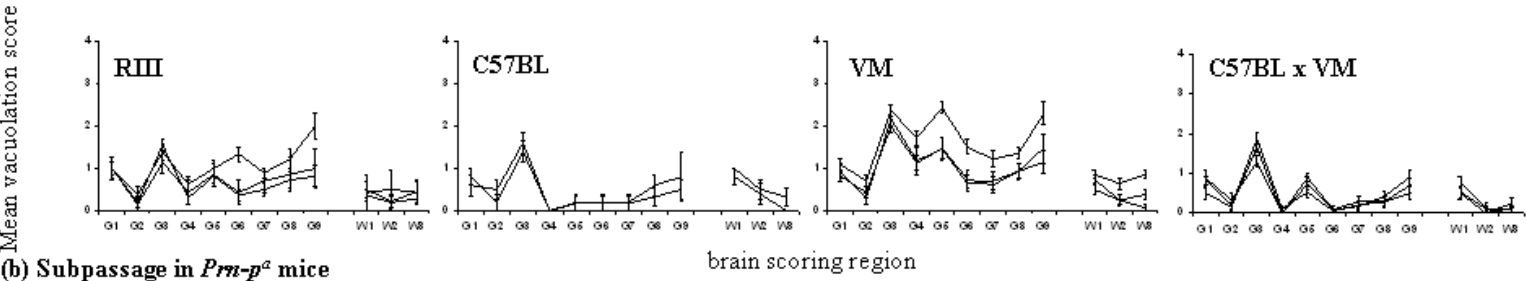
In general, there was little difference in the pattern or severity of vacuolar pathology between first and second mouse-to mouse passages. Overall, the pattern of vacuolar pathology in each mouse line was consistent with that observed on primary transmission to the same mouse line. However, VM mice with MM1 homogenate passaged in *Prn-p^a* mice showed a lesion profile more reminiscent of primary transmission from sCJD MV1 brain tissue to VM mice (Figure 4.3). Vacuolar pathology was characterised by a distinct and consistent targeting of mild-moderate vacuolation in the superior colliculus (brain scoring region G3) in all groups. There was also a widespread involvement of the thalamus (scoring region G5) and the cerebral cortex (scoring region G8 and G9). In VM mice challenged with isolates passaged in *Prn-p^b* mice, most of which died with clinical disease, vacuolation scores were consistently higher than in all other experimental groups, with widespread moderate vacuolation throughout the scoring regions of the brain. Although not all

profiles were based on mice at clinical end-point of disease, they clearly show a similarity in lesion distribution between the three sCJD MM1 brain homogenates and confirm that lesion profiles were similar irrespective of the *Prn-p* genotype of the mice through which the sCJD brain homogenate was passaged.

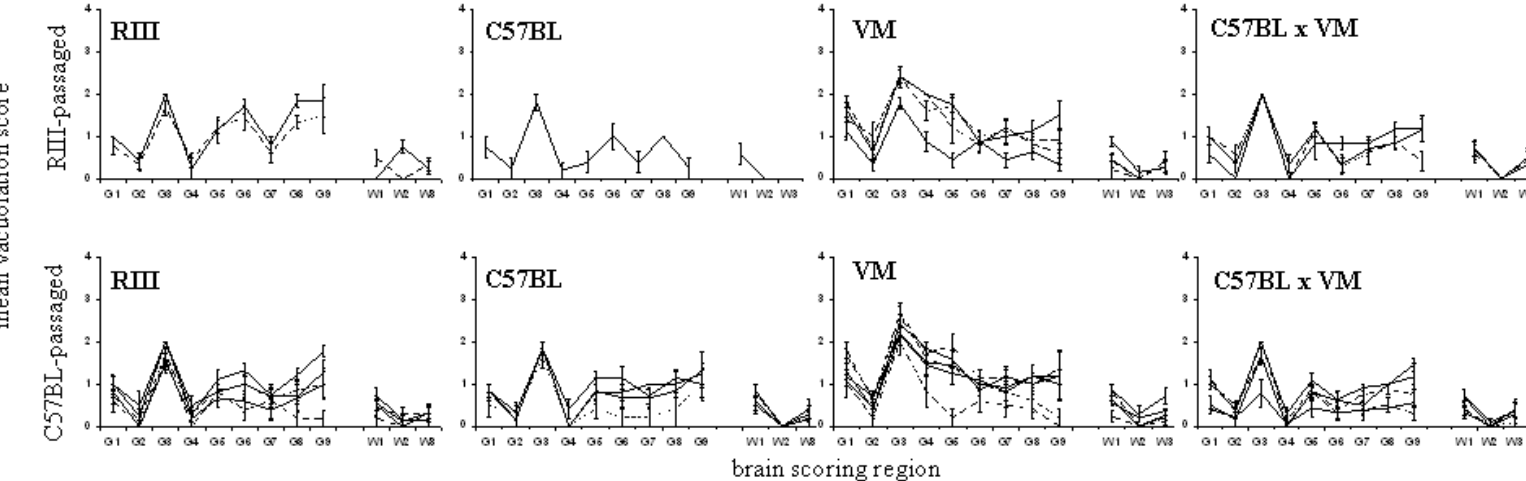
Figure 4.26: Lesion profiles for mice following sub-passage of sCJD MM1

Lesion profiles are constructed from a minimum of five mice with a positive score for TSE associated vacuolar pathology. Vacuolation was scored at nine specified grey (G) matter regions and three white (W) matter regions of the mouse brain. Lesion profiles shown are for all mouse lines in the (a) the primary transmission of brain homogenate from three sCJD MM1 cases (CJD 1, CJD 2 and CJD 5) and the subsequent first and second mouse-to-mouse passage of the same three brain isolates in (b) *Prn-p^a* and (c) *Prn-p^b* mice. Lesion profiles are shown with standard error bars (\pm S.E.M). (Solid lines) represent first mouse-to-mouse passage, (Dashed lines) second mouse-to-mouse passage.

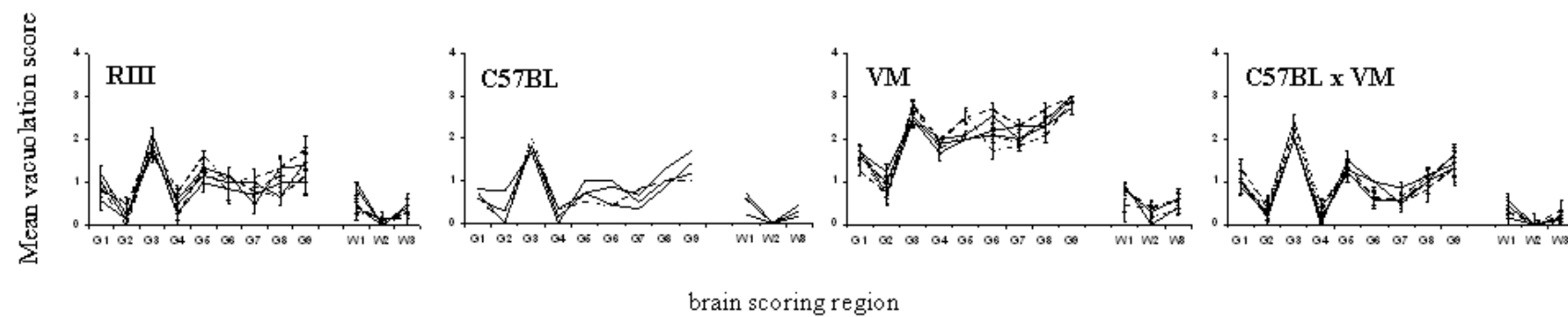
(a) Primary transmission



(b) Subpassage in *Prn-p^a* mice



(c) Subpassage in *Prn-p^b* mice



4.4.3 Deposition of PrP in the mouse brain

Patterns of PrP deposition in the mouse brain were investigated following the first and second mouse-to-mouse passage of isolates derived from a single sCJD MM1 (CJD 1) frontal cortex sample. Immunohistochemistry was carried out on 5µm formalin fixed, formic acid treated, brain sections from all three recipient mouse lines (RIII, VM and C57BL) and the C57BL x VM cross using the anti-PrP antibody 6H4 in combination with the CSA detection method as previously described (Chapter 2, section 2.6.4). In each experiment, three mice from each mouse line were analysed. In the selection of mice for immunohistochemical analysis, mice which had received a positive score for both clinical signs of disease and vacuolar pathology were preferentially selected. In the absence of clinically positive mice, mice which had received a positive score for vacuolar pathology with the longest survival time were selected. Results from the immunohistochemical analysis of PrP deposition in the brains of mice after sub-passage are shown in Table 4.9. Not all experimental groups showed the deposition of PrP within the mouse brain after the first passage in mice. However, PrP was detected in all mouse lines after a further passage in the mice. The patterns of PrP accumulation in the mice are detailed below.

Table 4.9: Deposition of PrP in mice following sub-passage of sCJD MM1

Donor tissue source	Recipient mouse line				
	Passage	RIII	C57BL	VM	C57BL x VM
CJD 1 RIII	1 st	*2/3	‡0/3	*1/3	†3/3
	2 nd	§2/3	†2/3	†3/3	†3/3
CJD 1 C57BL	1 st	*1/3	‡0/3	§0/3	†3/3
	2 nd	‡3/3	‡3/3	†3/3	†3/3
CJD 1 VM	1 st	*0/3	*0/3	*2/3	†3/3
	2 nd	‡2/3	†3/3	*3/3	†3/3

* mice with a positive score for vacuolar pathology sacrificed with clinical disease

† mice with a positive score for vacuolar pathology in the absence of clinical signs of a TSE

‡ all mice positive for vacuolar pathology, one mouse showing clinical TSE disease

§ all mice positive for vacuolar pathology, two mice showing clinical TSE disease

4.4.3.1 Sub-passage in *Prn-p^a* mice

Immunohistochemical labelling for the prion protein showed PrP deposition in the brains of all C57BL x VM mice examined after the first mouse-to-mouse passage, but in only a proportion of the brains of the RIII and VM mice. No evidence of PrP deposition was found in the brain of any C57BL mouse examined after first passage through either of the *Prn-p^a* mouse line (Table 4.9). In contrast, immunohistochemical analysis of the mice after a further serial passage in the *Prn-p^a* mice showed PrP deposition in the brain of almost all mice examined. The detection of PrP in the brains of C57BL mice after the second serial passage indicates the presence of infectivity in the C57BL mice after the first passage in the absence of any detectable levels of PrP by immunohistochemical analysis. The deposition and targeting of PrP pathology in the mice was closely similar to that observed in the primary transmission of sCJD MM1, with a restricted pathology of small granular deposits of PrP consistently targeting the vestibular nucleus of the medulla (Figure 4.27a and b) and the dLGN (Figure 4.27c and d) in all mouse lines. Deposition in the medial habenular nucleus (MHb), an area consistently targeted in the primary transmissions, was observed only in the VM mouse line (Figure 4.27e and f). In general, PrP showed a more widespread and intense pattern of deposition after second passage in mice (Figure 4.28a – d) with further PrP deposits found within the thalamus, pons and superior colliculus in a proportion of the mice examined (Figure 4.28e and f). There was no evidence of amyloid plaques in any of the mice examined.

Figure 4.27: PrP deposition after serial passage of sCJD MM1 in *Prn-p^a* mice

Faint granular deposits of PrP targeting the vestibular nucleus of the medulla in (a) RIII and (b) VM mice after the first passage in RIII mice. Granular deposition of PrP in the dorsal lateral geniculate nucleus (dLGN) of C57BL x VM mice following the first passage in (c) RIII and (d) C57BL mice. Granular deposits of PrP within the medial habenular nucleus (MHb) in VM mouse following the (e) first and (f) second mouse passage in RIII mice. Sections shown were labelled with the monoclonal anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification x400.

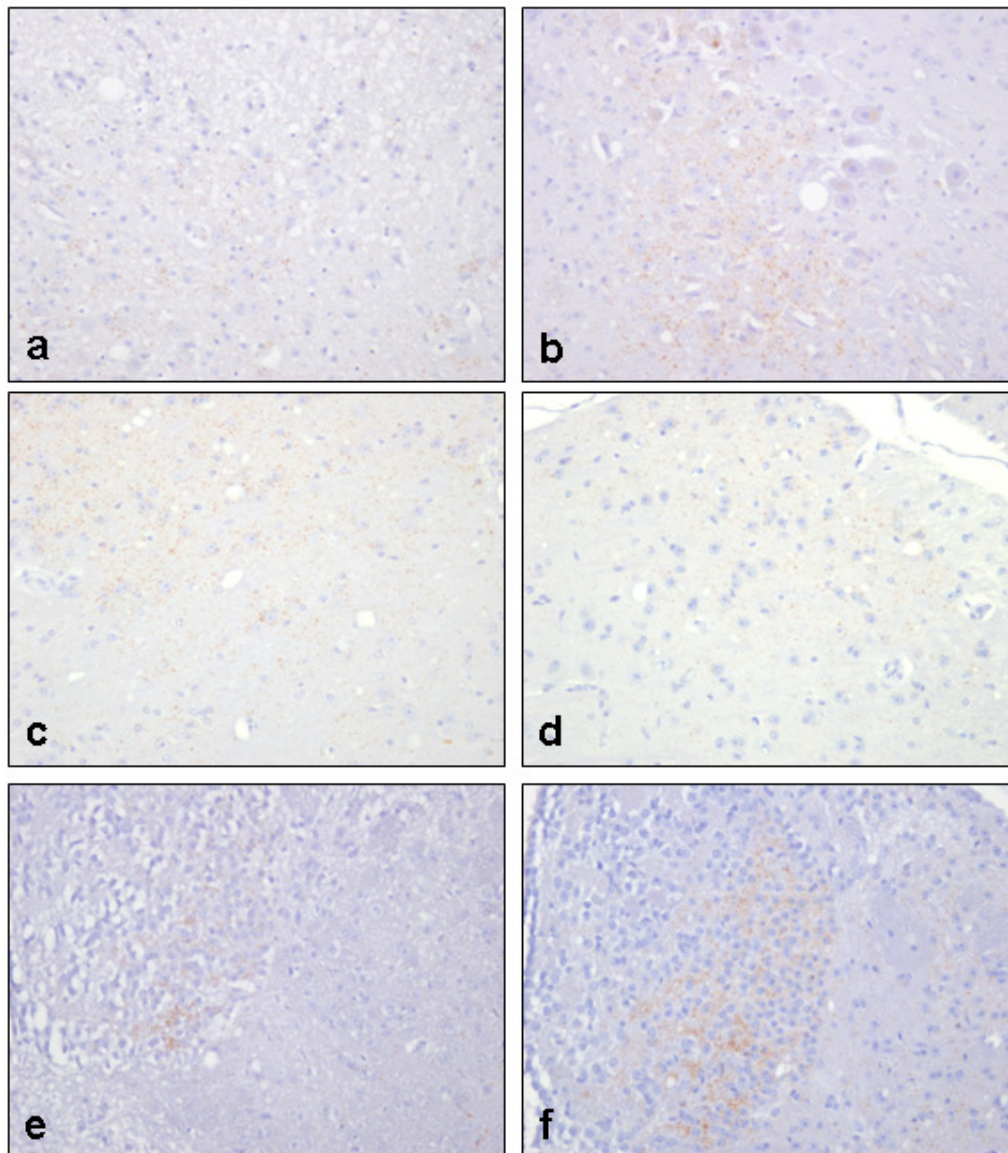
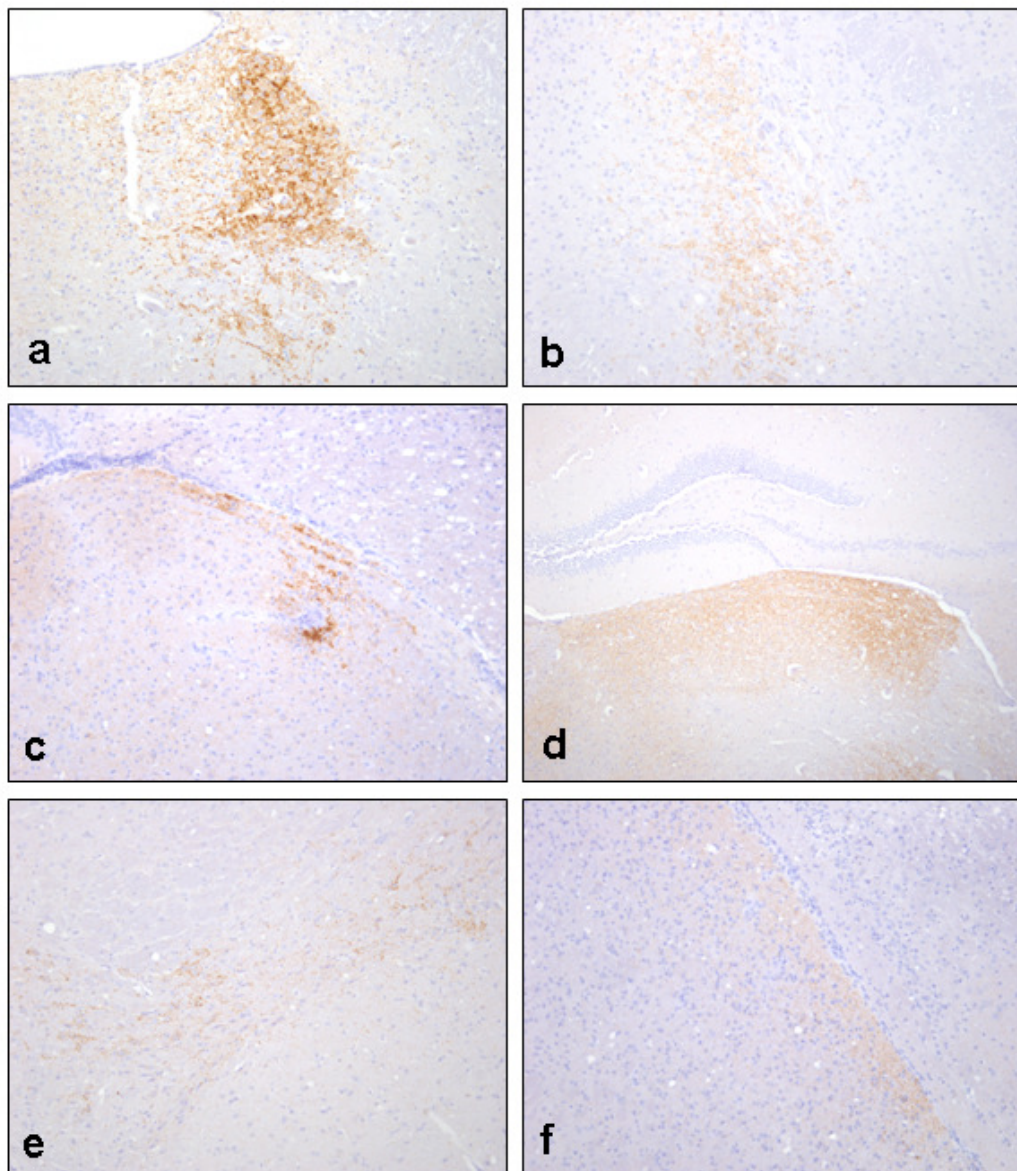


Figure 4.28: PrP deposition after second serial passage of sCJD in *Prn-p^a* mice

Immunohistochemistry for the prion protein in mice following second mouse-to-mouse passage of sCJD MM1 brain isolates in *Prn-p^a* mice. Strong peri-neuronal labelling for PrP in the vestibular nucleus of the medulla in (a) a VM mouse after passage in the RIII mouse line and (b) a C57BL x VM mice after passage through C57BL mice. (c) Strong punctate labelling in the dorsal lateral geniculate nucleus (dLGN) in a VM mouse after passage in the RIII mouse line. (d) Widespread synaptic labelling in the dLGN in a C57BL after passage through C57BL mice. (e) Peri-neuronal staining for PrP throughout the pons of an RIII mouse after passage through RIII mice. (f) Faint granular deposition of PrP around vacuolation in the superior colliculus in a C57BL mouse after passage through the C57BL mouse line. Sections shown were labelled with the monoclonal anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x100 (d), x200 (a, b, c, e, f).

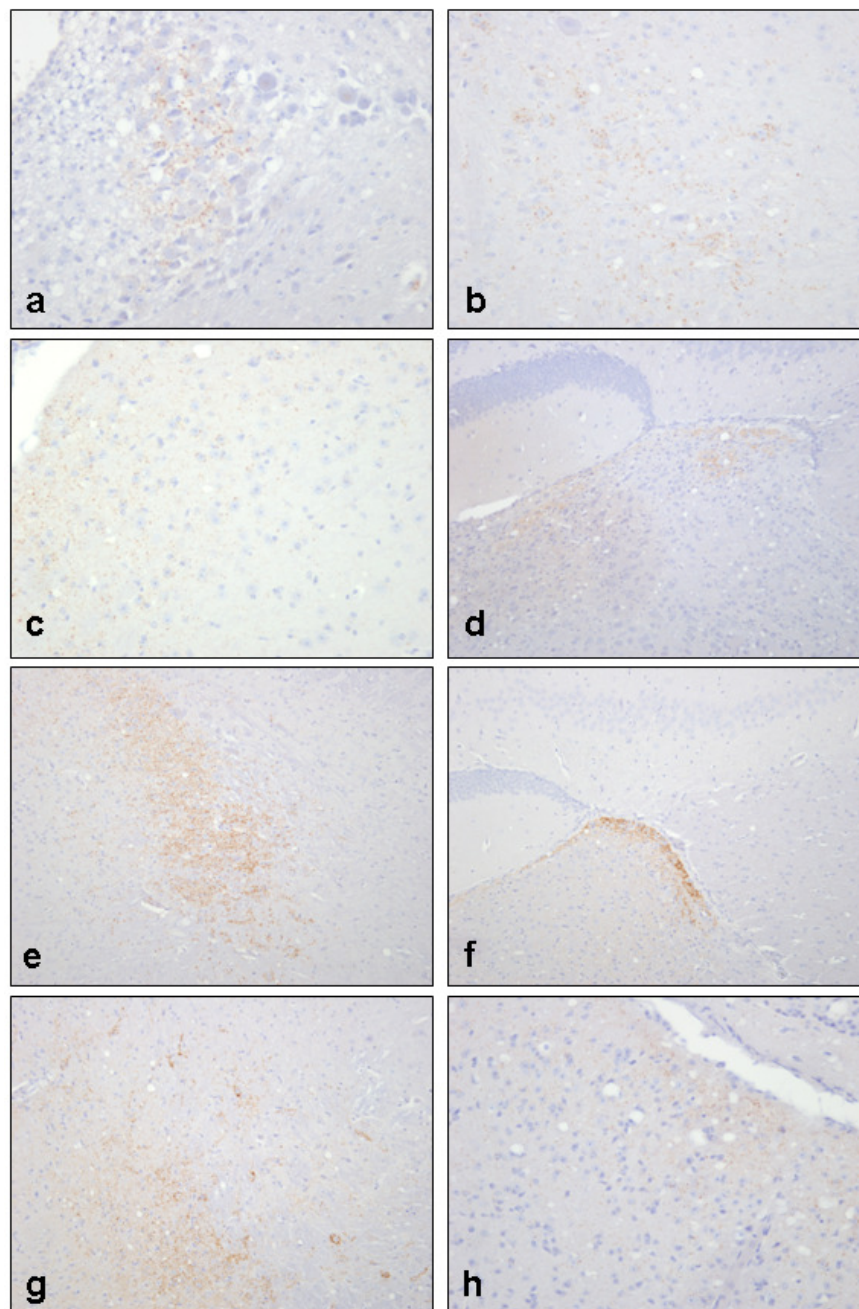


4.4.3.2 Sub-passage in *Prn-p^b* mice

Immunohistochemical staining showed no evidence of PrP deposition in the brain of any *Prn-p^a* (RIII and C57BL) mouse examined after the first passage of sCJD brain isolates through VM mice. However, examination of the brains of RIII and C57BL mice following a further VM-passage showed PrP deposition in the brain of almost all mice tested (Table 4.9). These results support findings from the serial passage of sCJD in *Prn-p^a* mice which indicated that infectivity may be present in the absence of any PrP accumulation in the mice as judged by immunohistochemical analysis. In contrast, PrP deposition was observed in almost all *Prn-p^b* (VM) and *Prn-p^{ab}* (C57BL x VM) mice after the first and second passage in VM mice (Table 4.9). The deposition and targeting of PrP pathology in the mice was closely similar to that observed in the same mouse lines in the primary transmission of sCJD MM1 and after serial passage in *Prn-p^a* mice, with a restricted pathology of small granular deposits of PrP consistently targeting the vestibular nucleus of the medulla (Figure 4.29a and b) and the dLGN (Figure 4.29c and d). Overall, PrP pathology was more widespread following a further mouse-to-mouse passage with further PrP deposits found within the thalamus, pons and superior colliculus in a proportion of the mice examined (Figure 4.29e and f). In contrast to primary transmissions and passage through *Prn-p^a* mice, deposition of PrP^{Sc} in the MHb was not a feature in the mice challenged. There was no evidence of amyloid plaques in any of the mice examined.

Figure 4.29: PrP deposition after serial passage of sCJD MM1 in *Prn-p^b* mice

Immunohistochemistry for the prion protein in mice after a first (a-d) and second (e-h) mouse passage in *Prn-p^b* (VM) mice. Small granular deposits of PrP around neurons in the vestibular nucleus of the medulla in a (a) VM and (b) C57BL x VM mouse. Granular deposits of PrP in the dorsal lateral geniculate nucleus (dLGN) of a (c) VM and (d) C57BL x VM mouse. (e) Strong granular deposits of PrP in the vestibular nucleus of an RIII mouse. (f) Intense deposits of PrP^{Sc} staining the dLGN of a VM mouse. (g) Peri-neuronal staining for PrP throughout the pons of C57BL x VM mouse. (h) Faint granular deposition of PrP in the superior colliculus in a C57BL x VM mouse. Sections shown were labelled with the monoclonal anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x200 (d, e, f, g), x400 (a, b, c, h)



4.4.4 Biochemical isotyping of PK resistant PrP^{Sc}

This thesis has confirmed the conservation of PrP^{res} type in mice following the primary transmission of sCJD MM1 from human brain homogenate. To determine whether the PrP^{res} type observed in sCJD MM1 brain homogenate is maintained on subsequent mouse-to-mouse passage, characterisation of PrP^{res} was carried out on all three mouse lines (RIII, C57BL and VM) and the C57BL x VM mice following serial passage in *Prn-p^a* (C57BL) and *Prn-p^b* (VM) mice. PrP^{res} types were analysed from the first and second mouse-to-mouse passage of the isolates derived from a single sCJD MM1 cerebellar (CJD 2) sample. Western blot analysis was performed on a minimum of two mice from each experimental group, using the standard Western blot protocol used in the diagnostic typing of human TSEs and in the detection and typing of PrP^{res} in mice following primary transmission of brain homogenate from sCJD subgroups (Chapter 2, section 2.7). All mice analysed had shown evidence of TSE-associated vacuolar pathology. Western blot analysis was carried out on 5µl samples of a 10% brain tissue weight to buffer volume (w/v) unless otherwise stated. As described previously, mouse samples were run alongside a sCJD MM1 diagnostic brain reference standard and a vCJD diagnostic brain reference standard serving as controls for both fragment mobility and glycoform ratio.

4.4.4.1 Sub-passage in *Prn-p^a* mice

PrP^{res} was readily detected in CNS extracts from all mice analysed following passage in C57BL mice. The PrP^{res} detected in the mouse extracts showed a glycosylation pattern and electrophoretic mobility of the unglycosylated form of the protein closely resembling that detected in the primary transmission of sCJD MM1 to the same mouse lines and that seen in sCJD MM1 patients (Figure 4.30). The PrP^{res} banding pattern was similar in mice of all *Prn-p* genotypes and showed no observable difference in glycosylation pattern or in the mobility at the unglycosylated band between the first and second mouse-to-mouse passage (Figure 4.30). These results confirm the findings from the primary transmission of sCJD MM1 brain homogenate, which demonstrated that the biochemical properties of PrP^{res} from this subgroup are maintained on primary transmission and on subsequent passage in mice.

4.4.4.2 Sub-passage in *Prn-p^b* mice

PrP^{res} was readily detected in all mouse CNS extracts examined after passage in VM mice. Consistent with the primary transmission data and that of serial passage in *Prn-p^a* mice, Western blotting of CNS extracts demonstrated a PrP^{res} banding pattern closely resembling that seen in sCJD MM1 cases and in mice following experimental challenge with sCJD MM1 brain homogenate, with a molecular weight at the unglycosylated fragment of approximately 21kDa (Figure 4.31). Furthermore, the PrP^{res} type in the mice was closely similar to that observed in the same mouse lines after serial passage in *Prn-p^a* mice (Figure 4.30). The similarity in the PrP^{res} type in VM mice infected with isolates passaged in *Prn-p^a* and *Prn-p^b* mice shows no evidence of the two distinct mouse-passaged strains that were inferred from the incubation period data. Again there were no observable differences in the PrP^{res} type between the first and second mouse-to-mouse passage (Figure 4.31). These results confirm that the type 1 PrP^{res} seen in the original sCJD inoculum is maintained on primary transmission and on subsequent serial passage in VM mice.

Figure 4.30: Western blot analysis of PrP^{res} in sCJD MM1 brain isolates passaged in *Prn-p^a* mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after the 1st and 2nd mouse-to-mouse passage of brain isolates in C57BL (*Prn-p^a*) mice. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).

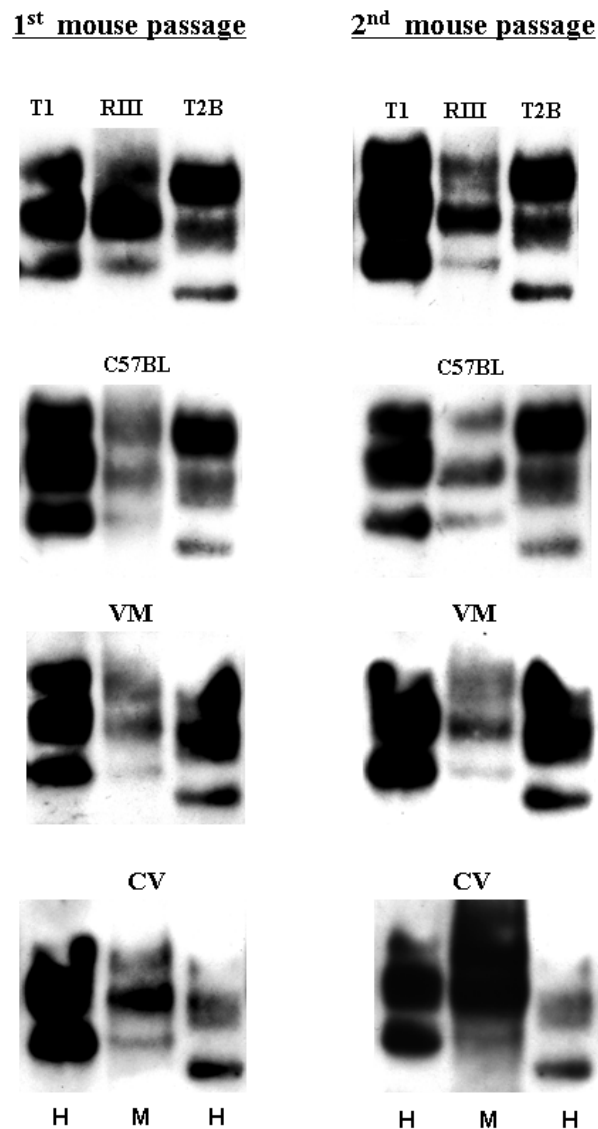
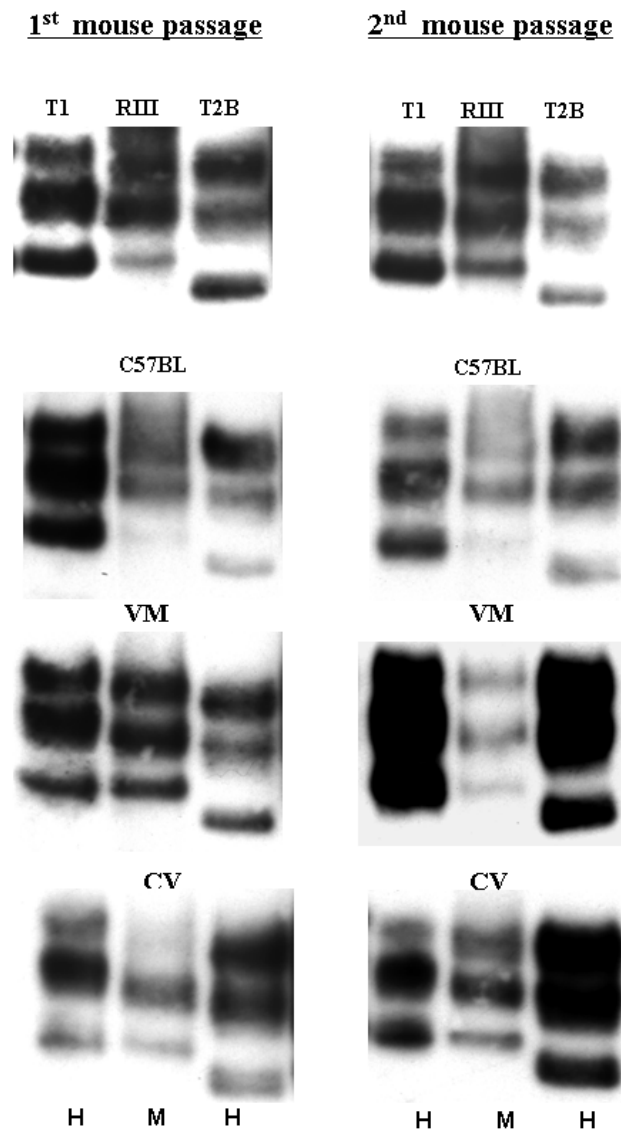


Figure 4.31: Western blot analysis of PrP^{res} in sCJD MM1 brain isolates passaged in *Prn-p^b* mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after the 1st and 2nd mouse-to-mouse passage of brain isolates in VM mice. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).



4.5 Results Summary

In addressing the question of whether different strains of agent are present within sCJD, this chapter examined the transmission characteristics of all six sCJD subgroups, as classified according to PrP^{res} type and codon 129 *PRNP* genotype, after inoculation into panels of wild-type mice. Results from the primary transmission and the subsequent mouse-to-mouse passages have shown that all three mouse lines (RIII, C57BL and VM) and the C57BL x VM mice are susceptible to infection with sCJD; however, not all subgroups of sCJD examined transmit efficiently in the mouse panel. Transmission was most efficient from the sCJD MM1, MV1 and MM2 brain homogenate. In contrast, transmission of disease from sCJD MV2, VV1 and VV2 brain homogenates was rarely observed. Sporadic CJD in wild-type mice is characterised by the following features;

- Clinical signs of a neurological disease are rare in wild-type mice after primary challenge with sCJD subgroups.
- Histological and biochemical analysis, confirms a TSE in mice dying with intercurrent illness after challenge with all six sCJD subgroups.
- Transmission as determined by the presence of vacuolar pathology in the mouse brain and the detection of PrP deposition in the brain by immunohistochemistry and Western blotting is most efficient in mice inoculated with sCJD MM1, MV1 and MM2 brain homogenates.
- Lesion profiles show a similar pattern of vacuolar pathology between mouse lines of different *Prn-p* genotypes inoculated with different sCJD MM1 and MV1 isolates. This pattern of vacuolar pathology showed subtle differences from mice inoculated with a single sCJD MM2 isolate.
- Immunohistochemistry shows a precise targeting of PrP pathology in the brains of infected mice. However, PrP pathology is not a major feature of sCJD in wild-type mice.
- sCJD cases with a PrP^{res} type 1 in the brain show conservation of PrP^{res} type on primary transmission and after serial passage in mice.

- PrP^{res} typing indicates that sCJD with type 2 PrP^{res} detected in the brain inoculum do not always show conservation in PrP^{res} type when experimentally transmitted to mice.
- Serial passage of the sCJD MM1 agent in *Prn-p^a* and *Prn-p^b* mice results in the isolation of two distinct mouse-passaged strains, distinguishable by whether or not they produce clinical disease in VM mice.

4.6 Discussion of experimental results

The aim of this chapter was to characterise the agent strain in sCJD by transmissions to wild-type mice. Using incubation times, patterns of TSE associated vacuolar pathology and detection of PrP by immunohistochemistry and Western blot in mice, similarities and differences between the six sCJD subgroups have emerged. Overall, the data presented in this thesis implies the existence of at least two distinct strains of sCJD, which can be distinguished by subtle differences in their transmission properties following inoculation in panels of wild-type mice. The lack of transmission for other sCJD subgroups suggests that they could represent a further third strain of agent.

4.6.1 Preclinical TSE disease in sCJD inoculated wild-type mice

In previous studies examining the transmission properties of sCJD in wild-type mice, clinical signs of disease were rarely observed, suggesting that a substantial ‘species barrier’ exists between the donor (human) and recipient species (mouse) (Bruce et al., 1997; Hill et al., 1997; Asante et al., 2002; Wadsworth et al., 2008). In support of these findings, this thesis confirms that the development of clinical TSE disease in wild-type mice is rare following inoculation of brain homogenate from sCJD MM1, MV1, MV2, VV1 and VV2 cases, consistent with the existence of a ‘transmission barrier’. Therefore, in the experimental challenge of sCJD brain homogenate to wild-type mice, incubation period data is not able to distinguish between sCJD MM1, MV1, MV2, VV1 and VV2 cases and is therefore not a relevant strain typing parameter for these five sCJD subgroups. In contrast, clinical TSE disease was observed in seven out of 19 RIII mice inoculated with a single sCJD MM2 brain homogenate, suggesting that the sCJD MM2 subgroup may be distinguished from

other sCJD subgroups on the basis of development of clinical disease in a proportion of RIII mice challenged. Inoculations from the sCJD MM2 subgroup are discussed in detail later in this discussion (Chapter 4, section 4.6.3)

The lack of clinical TSE disease in mice inoculated with sCJD MM1, MV1, MV2, VV1 and VV2 brain homogenate, does not necessarily indicate a failure to transmit disease from these five subgroups. Examination of the brain for TSE associated vacuolar pathology, PrP deposition and biochemical analysis of PrP^{res} provided evidence of TSE disease in a proportion of mice dying with intercurrent disease. This confirms the successful transmission of TSE infectivity from these five sCJD subgroups in the wild-type mouse panel. However, it is difficult to determine whether these mice are at a preclinical stage in which clinical signs would develop had the mice survived long enough, or whether these mice have a subclinical disease in which clinical signs do not develop but the mice would remain asymptomatic carriers of TSE infection however long they lived. The observations of clinical disease in 12 mice (from the 580 surviving over 250dpi) inoculated with sCJD MM1 homogenates and also in seven (from the 91 surviving over 250dpi) inoculated with sCJD MM2 homogenate shows that, although rare, wild-type mice are capable of developing clinical TSE disease within their life spans. This suggests that preclinical disease in the sCJD inoculated mice is a more likely explanation.

The presence of TSE-specific vacuolar pathology and the detection of PrP^{res} in the brains of mice, confirmed the successful transmission of a TSE from all sCJD subgroups; however, the efficiency of transmission, as assessed by the numbers of mice showing pathological or biochemical evidence of a TSE varied according to the individual brain homogenate and was used to further distinguish between the different sCJD subgroups. Such differences in the transmission efficiency in the different sCJD subgroups are not likely to be a result of species mis-matching, as the donor and recipient species are consistent throughout this series of experiments. Therefore, these findings, support recent proposals that ‘transmission barrier’ rather than ‘species barrier’ may be a better term to describe the variation in the transmission efficiency between different TSE inocula and different species (Hill et

al., 2003). It is more likely that the differences found were a result of differences in the individual inocula from the same donor species. Furthermore, the ‘species barrier’ effect is comparative based on the difference observed in the incubation period between the primary transmission of an isolate and its subsequent passage in the new host species. Usually, but not always, the efficiency of transmission increases on subsequent serial passage, which is represented by a decrease in the incubation period. This incubation period difference is thought partly to be a result of increased efficiency of replication in the new host and partly a result of agent strain selection. Surprisingly there does not appear to be any observable increase in the efficiency of sCJD agent transmission, at least for the *Prn-p^a* passages, where they resemble the primaries, indicating that there is little or no adaption to the new species. This is further evidence that the difficulties in sCJD transmission may be determined by agent strain rather than by a mis-match between species. It may simply be that sCJD strains are not replicated efficiently in these wild-type mice.

4.6.2 sCJD MM1/MV1 agent strain

Few if any differences were observed in transmission properties between brain homogenates from sCJD MM1 and MV1 cases. This suggests that the MM1 and MV1 subgroups represent a distinct strain of agent within sCJD. This finding is consistent with the current clinical and pathological classification of sCJD in which the MM1 and MV1 subtypes are grouped together as the ‘classical’ forms of sCJD (Parchi et al., 1999). Vacuolar degeneration typical of TSE infection was seen in a high percentage of mice following inoculation with both MM1 and MV1 homogenate, indicating that transmission of sCJD is most efficient from these two subgroups. Lesion profiles showed a similar pattern of TSE-specific vacuolation between with the 10 sCJD MM1 cases and the three sCJD MV1 cases, consistent with the proposal that these two subgroups share similar strain properties. PrP pathology, as judged by immunohistochemistry was limited in mice following inoculation with sCJD MM1 and sCJD MV1 isolates. This may be an indication that PrP accumulation in the mice occurs at a later stage in the progression of the disease, after the appearance of vacuolar degeneration. Alternatively, PrP pathology (as detectable by immunohistochemistry) may not a major pathological feature of sCJD in

wild-type mice. As mice were not sacrificed as a result of clinical TSE disease this is difficult to determine in the primary transmission series. However, immunohistochemical analysis of PrP deposition in mice sacrificed with clinical TSE following sub-passage of sCJD MM1, showed a restricted pattern of PrP deposition in the brain, supporting the proposal that PrP pathology may not be a major feature of sCJD in wild-type mice. Western blot analysis of CNS tissue from mice of all *Prn-p* genotypes after inoculation with sCJD MM1 and MV1 homogenates produced a PrP^{res} type that was similar to the type 1 documented in the sCJD inocula. No observable difference was found in the molecular weight at the unglycosylated PrP^{res} or the PrP^{res} glycosylation ratio in mice inoculated with either sCJD MM1 or MV1 brain homogenate. This is further supporting evidence indicating a single strain of agent associated with the sCJD MM1 and MV1 subgroups.

4.6.3 sCJD MM2A agent strain

Certain differences were seen in the transmission properties of the two sCJD MM2 cases. In one of the MM2 cases, patient CJD 12, transmission properties showed subtle differences from those of the other five sCJD subgroups suggesting that the sCJD MM2 subgroup may represent a second strain of sCJD. In contrast to the MM1, MV1, MV2, VV1 and VV2 subgroups, clinical TSE disease was evident in a proportion of RIII mice following inoculation with inoculum from patient CJD 12. The resulting incubation period was 736 ± 9 days. Although titres of infectivity in the primary inocula used in this series of transmissions are not known, one possible explanation for the appearance of clinical disease in this experimental group may simply be that titres of infectivity were higher in this inoculum compared to the other sCJD inocula or that the sCJD MM2 subtype is associated with high titres of infectivity. However, there was no evidence of this when comparing the intensity of the Western blot banding pattern from this MM2A case with that of the other 26 sCJD inocula. Lesion profiling and PrP^{res} typing of CNS tissue of the mice inoculated with this sCJD brain inoculum, provided further evidence that the sCJD MM2 subtype represents a distinct strain of sCJD. Lesion profile from RIII mice produced a subtly different profile to that observed in RIII mice following challenge with inocula from the MM1 and MV1 brain isolates. Furthermore, the lack of any

vacuolar pathology and lesion profile in the VM and C57BL x VM mice also distinguished the sCJD MM2 subtype from the sCJD MM1 and MV1 subgroups. Western blot analysis of CNS tissue from RIII, VM and C57BL x VM mice showed conservation in the electrophoretic mobility and glycosylation pattern of the PrP^{res} type 2A as detected in the inoculum from this CJD case, further distinguishing this sCJD MM2 brain isolate from the sCJD MM1 and MV1 subgroups.

Experimental data from a second sCJD MM2 (patient CJD 11) inoculation was limited to two mouse lines (RIII and VM). Mice inoculated with this brain homogenate showed no evidence of the clinical TSE disease that developed in the RIII mice following challenge with inoculum from CJD 12. In this respect, this sCJD MM2 isolate resembled the sCJD MM1 and MV1 brain isolates. Furthermore, lesion profiling carried out with three RIII mice showing vacuolar pathology showed a pattern more consistent to that found with MM1 and MV1 isolates. Although frozen mouse tissue was unavailable in this experiment, this suggests that this sCJD MM2 subtype transmits to the mice with a phenotype more commonly associated with sCJD MM1 and MV1 cases rather than that of the other sCJD MM2 inoculum. One explanation could be that this sCJD MM2 brain isolate contained a minority type 1 component that was undetected by PrP^{res} typing of the inoculum. It may be that the minority type 1 is preferentially selected by the recipient mice over the more abundant type 2 detected in the inoculum. In support of this explanation, this particular sCJD brain is known to contain both PrP^{res} type 1 and type 2, as judged by analysis of a large cortical specimen used to prepare a sCJD reference standard by the National Institute of Biological Standards and Control (Minor et al., 2004). Therefore in this thesis, the results from CJD 12 may be more representative of transmission from a 'pure' sCJD MM2 isolate in mice than those obtained from CJD 11 in which a mixture of PrP^{res} types are likely. It would be interesting to re-examine these inoculum with an antibody specifically raised against PrP^{res} type 1, to determine whether this hypothesis is correct.

4.6.4 sCJD MV2A, VV1 and VV2A agent strain

Evidence of a TSE in mice following challenge with sCJD MV2, VV1 and VV2 brain isolates was rare, in any of the *Prn-p* genotypes investigated. Inoculation of three sCJD MV2 isolates resulted in one mouse (from the 157 surviving over 250dpi) producing evidence for transmission of disease, with vacuolar degeneration and PrP^{res} detected in the brain. Inoculation of four sCJD VV1 isolates similarly resulted in only a single mouse (from the 235 surviving over 250dpi) showing evidence of transmission with TSE associated vacuolar degeneration and PrP^{res} detected in the brain. In the case of the sCJD VV2 subgroup, inoculation with five different VV2 sources gave evidence of transmission in one mouse (from the 341 surviving over 250dpi), again with vacuolar pathology and PrP^{res} detected in the brain. This lack of transmission distinguished the brain inocula from these three sCJD subgroups from that of sCJD MM1, MV1 and MV2. One possibility for the lack of transmission in these sCJD subgroups is that lower levels of infectivity are associated with the frontal cortex tissue sampled from the MV2, VV1 and VV2 cases. However, this is unlikely, as Western blotting on extracts of these inocula showed comparable levels of PrP^{res} to those observed in extracts of the sCJD MM1, MV1 and MM2 inocula (chapter 3). If PrP^{res} does correspond to infectivity levels as has been suggested (Prusiner, 1998; Weissmann, 2004; Aguzzi et al., 2008), this implies that similar levels of infectivity were present in the different inocula tested and that the differing transmission characteristics most likely derive from strain-related properties.

In any experimental transmission, a failure to transmit disease is often an indication of a 'species barrier' between the donor tissue and the recipient species. However, this thesis has confirmed that some sCJD subtypes do successfully transmit disease. Furthermore, findings from the subsequent mouse passage of sCJD MM1 brain isolates suggest that there may be no 'species barrier' present between the wild-type mice and the sCJD brain isolates. Therefore, it is possible, as has been discussed earlier (Section 4.6.1), that the sCJD MV2, VV1 and VV2 subgroups are associated with different 'transmission barriers' to that of the sCJD MM1, MV1 and MM2. Results would also suggest that the 'transmission barrier' to the development of disease from sCJD MV2, VV1 and VV2 subgroups is more difficult to overcome

than that of sCJD MM1, MV1 and MM2. Further evidence for the existence of different ‘transmission barriers’ in the experimental transmission of CJD is provided in Chapter 5 where, the efficient transmission of vCJD with the development of clinical disease in the same mouse panel is distinct from all sCJD subgroups.

The lack of transmission from these sCJD subgroups may also be evidence that these sCJD subgroups (MV2, VV1, and VV2) represent a further sCJD strain. This was also suggested in a comparable investigation on the transmission of the different sCJD subgroups in the bank vole (Nonno et al., 2006). A comparison of the findings from this thesis and the study in bank voles is discussed in Chapter 7, section 7.2.1. However, the possibility that these three sCJD subgroups could represent more than one strain of agent which can not be distinguished in this mouse model can not be discounted. Evidence that there are further strains of agent within these three sCJD subgroups has been supported through recent findings from a comparative study analysing the transmission properties of all six sCJD subgroups in humanised transgenic mice (Bishop, 2008; Bishop et al., 2010). Analysis of the transmission properties in this transgenic mouse model are examined in detail in Chapter 7 and indicate that two strains of agent are propagated in these transgenic mice following experimental challenge with sCJD MV2, VV2 and VV1; one associated with the MV2 and VV2 subgroups and a further strain associated with the sCJD VV1 subgroup.

4.6.4.1 Role of codon 129 genotype and PrP^{res} type in the transmission of sCJD in wild-type mice

A further explanation for the lack of transmission from sCJD MV2, VV1 and VV2 subgroups may reside in the mis-match between the *PRNP* codon 129 genotype of the donor inoculum and the corresponding *Prn-p* codon 128 in the recipient host (wild-type mice strains are all homozygous for methionine at the corresponding *Prn-p* codon 128). Although this may explain the ‘transmission barrier’ between the mice and the human sCJD VV cases, it does not provide an explanation for the difference in transmission properties found between sCJD MV1 and MV2 cases. Isolates prepared from MV1 sources transmit successfully in the mice whereas the MV2

isolates do not appear to. It is reasonable to suggest that such differences in the transmission properties may be related to agent strain. As both subgroups are heterozygous at *PRNP* codon 129, one possibility is that the strain-specific differences between sCJD subgroups may in part be attributed to the differences in the PrP^{Sc} conformation between these two sCJD subtypes. This is further supported by differences observed in the transmission properties between mice inoculated with sCJD MM1 and sCJD MM2 brain homogenate (Chapter 4, section 4.6.). This is consistent with the proposal that distinct TSE strains are associated with differences in PrP^{Sc} conformation and (Bessen and Marsh, 1992a; Bessen and Marsh, 1994; Collinge et al., 1996; Telling et al., 1996) and glycosylation (Collinge et al., 1996, Lasmezas et al., 1996, Hill et al. 1997). However, the question of whether the different PrP^{res} types alone could encode agent strain is still unclear.

Another possible explanation for the lack of transmission in these three sCJD subgroups (which would be difficult to determine within the bounds of this transmission series) is that there are differential allelic contributions in the formation of PrP^{Sc} in the heterozygotes. This proposal has been made in a recent study by Jones and co-workers using the cell-free PMCA method. The study examined the efficiency of sCJD PrP^{Sc}, from all six sCJD subgroups, as seeds in the conversion of PrP^C to PrP^{Sc} in substrates prepared from humanised transgenic mouse brain tissue of each of the *PRNP* codon 129 genotypes (Jones et al., 2008). Results from the PMCA conversion process confirmed that a complex relationship exists between the sCJD patients codon 129 genotype and the PrP^{res} type in the brain. In agreement with the findings of this thesis, efficiency in the conversion of PrP^C to PrP^{Sc} by PMCA was determined in part by compatibility in the seed/substrate *PRNP* codon 129 genotype. In this thesis the seed corresponds to the donor human tissue with the recipient mouse corresponding to the MM substrate. Jones and co-workers found that seeds from sCJD MM1, MV1 and MM2 amplified PrP^{Sc} in the MM substrate, whereas no amplification of PrP^{Sc} was observed from seeds of sCJD MV2, VV1 and VV2 cases in the MM substrate. By extending the investigations to include MV and VV substrates Jones provided evidence that seeds of sCJD VV can efficiently amplify PrP^{Sc} but only in VV substrate. This supports the proposition that the lack of

transmission in the sCJD VV cases in this thesis may be in part a result of mis-match in the *PRNP/Prn-p* genotypes. Furthermore, differences were found in the amplification of seeds from sCJD in MV patients. As described, sCJD MV1 cases behaved similarly to MM cases and amplify PrP^{Sc} in MM substrates. However, seeds from MV2 cases behaved similarly to VV cases, showing evidence of amplification only in VV substrates. This would suggest the PrP^{Sc} in sCJD MV1 cases is predominantly composed of PrP^{Sc} containing methionine at *PRNP* codon 129, whereas the PrP^{Sc} in MV2 cases consist predominantly of PrP^{Sc} containing valine at *PRNP* codon 129. These results are consistent with the findings of this thesis and might explain the differences observed in the transmission properties between the MV1 and MV2 cases.

4.6.4.2 Alteration in PrP^{res} type following experimental challenge of sCJD in wild-type mice

Biochemical typing of PrP^{res} in CNS tissue from the one RIII mouse showing positive transmission after inoculation with a sCJD MV2 brain homogenate, showed an electrophoretic mobility and glycosylation pattern resembling that seen in sCJD type 1 cases as well as that seen in RIII mice after inoculation with sCJD MM1, MV1 and VV1 (one positive mouse) inocula. Therefore, the PrP^{res} type associated with transmitted infection in this mouse is different in conformation to the PrP^{res} detected in the inoculum. Although caution is advisable in interpreting data based on a single mouse, such changes in the fragment size of unglycosylated PrP^{res} have also been reported in studies investigating the transmission properties of vCJD in transgenic mice expressing human PrP (Collinge et al., 1996; Hill et al., 1996; Asante et al., 2002; Beringue et al., 2008; Bishop et al., 2008; Bishop et al., 2010).

The detection of a different PrP^{res} type in one mouse following transmission of a single MV2 inoculum may be an indication of the existence of agent strain variation within some sCJD subtypes. Strain variation has been widely reported in TSEs and is best documented following transmission of TSE isolates in mice (Bruce, 2003). It is proposed that a change in species or even in the mouse line allows the selection of minor variant strains that replicate more efficiently in the host. In the case of the

sCJD MV2 transmission, it is possible that mouse transmission of sCJD has produced a strain associated with a different PrP^{res} conformation, one more commonly found with transmitted infection of cases of sCJD MM and MV type 1 and that detected in a single mouse following challenge with a VV1 isolate.

A further possibility for the detection of a different PrP^{res} type on sCJD transmission is outlined in the “conformational selection model” for cross-species transmission (Collinge, 1999). This model of TSE transmission proposes that within the mammalian PrP sequence there are a number of possible PrP^{res} conformations. The extent of any given ‘species’ or ‘transmission barrier’ is determined by the compatibility between the favoured or allowed conformations for PrP^{res} derived from the donor and recipient species. Substantial overlap between these possible PrP^{res} conformations between donor and recipient species would result in a low ‘transmission barrier’ compared to a high ‘transmission barrier’ between two species with fewer preferred PrP^{Sc} conformations in common. Overall, the lack of any clinical disease in the mice suggest a substantial ‘transmission barrier’ across the whole sCJD series; however, transmission of disease across all mouse lines is more readily achieved from sCJD cases with a PrP^{res} type 1 and in which there is a match at codon 129 on the PrP gene between the human inoculum and the recipient mouse. Therefore, in the transmission of sCJD from MV2 and VV2 cases there is an even greater ‘transmission barrier’ to overcome for the development of disease. The detection of a PrP^{res} type 1 in the single RIII mouse following challenge with an MV2 isolate may reflect the presence of a minority PrP^{res} type 1 component within the inocula that was undetected by conventional Western blotting using the anti-PrP antibody 3F4, an antibody shown to have quantitative limitations in the co-detection of type 1 and type 2 (Yull et al., 2006). Although, type 2 was the dominant PrP conformation detected within the inoculum, the presence of a minority component of type 1 within the inoculum would increase the compatibility between this preferred PrP^{res} type of the mouse and the incoming PrP^{res} type of the inoculum. This would result in transmission in the one mouse showing a different PrP^{res} type from that detected in the original inocula. However, this ‘conformational selection model’ does not hold true with the transmission of vCJD (Chapter 5), and to a much lesser degree

sCJD MM2 isolates, where infectivity is readily and efficiently transmitted in the mice from a human TSE with a type 2 PrP^{res}.

4.6.5 Confirmation of TSE infectivity in the absence of clinical TSE disease

The development of a TSE in a proportion of mice following subsequent mouse-to-mouse passage of three sCJD MM1 isolates confirmed the presence of infectivity within clinically asymptomatic mice after primary transmission. The presence of TSE infectivity in asymptomatic carriers is not a feature unique to sCJD transmissions in wild-type mice. Infectivity has been reported in the transmission of hamster scrapie in wild-type mice (Race and Chesebro, 1998; Race et al., 2001; Race et al., 2002) and in the transmission of vCJD and BSE in humanised transgenic mice (Asante et al., 2002; Hill and Collinge, 2002).

4.6.5.1 Isolation of distinct sCJD mouse-passaged strains following serial passage in different mouse Prn-p genotypes

Mean incubation period data was available for all VM experimental groups following passage of brain isolates in *Prn-p^b* mice. The development of clinical TSE disease in these VM groups is indicative of a reduction in the ‘transmission barrier’ which was present on primary transmission, and is a characteristic feature of subsequent sub-passage of TSE isolates within the same host species. In contrast, *Prn-p^b*-passaged isolates produced very little evidence of clinical disease in *Prn-p^a* mice. In fact, evidence of transmission as determined by clinical disease actually reduces after a further VM-passage. This suggests that the *Prn-p^a* mice may not necessarily be infected with the same strain of agent that is causing a clinical disease in the VM mice and whatever strain is infecting them is gradually being lost. Sub-passage of brain isolates in *Prn-p^a* lines showed very little evidence of a reduction of the ‘transmission barrier’ which was evident on primary transmission. In fact, sub-passage of brain isolates in *Prn-p^a* mice suggests that there may be no transmission barrier between sCJD and wild-type mice. Incubation periods, if present were long, and evidence of transmission reduced following a further *Prn-p^a* passage. These findings may be a reflection of lower levels of infectivity present in the mouse brain when compared to the original human sCJD since the majority of sCJD inoculated

mice, including those selected for serial passage were sacrificed before the development of a clinical TSE disease. Alternatively it may be that the sCJD isolates may not have adapted in the mouse lines.

The differences found in the clinical disease in VM mice following passage of brain isolates in either *Prn-p^a* and *Prn-p^b* mice, implies the isolation of two distinct mouse-passaged strains of sCJD MM1, selected according to the *Prn-p* genotype of host used for passage. Such a divergence of strains has been seen for a large number of TSE isolates from a range of different species. Importantly, transmission properties in the two strains isolated by VM mice in this investigation are unlike any previously isolated from any other source including vCJD (Moir Bruce, personal communication).

4.6 Chapter conclusion

This study has identified two distinct strains of sCJD by comparison of their transmission properties in wild-type mice; one associated with the MM1/MV1 subgroups and another associated with the sCJD MM2 subgroup. The lack of transmission from the sCJD MV2, VV1 and VV2 inocula in the same mouse panel implies that these three subgroups may represent a further sCJD strain or sCJD strains. The identification of these distinct sCJD strains are in agreement with that of previous studies investigating the transmission properties of sCJD in humanised transgenic models (Asante et al., 2002; Korth et al., 2003; Taguchi et al., 2003; Bishop, 2008; Bishop et al., 2010) and bank voles (Nonno et al., 2006) and appear to largely concur with the current clinico-pathological classification of sCJD (Parchi et al., 1999).

Chapter 5: Characterisation of the variant Creutzfeldt-Jakob agent by transmission to wild-type mice

5.1 Objective

1. To characterise the vCJD agent strain after primary transmission of human brain tissue in panels of wild-type type mice using the standard strain typing properties of incubation period and lesion profiles, plus a full immunohistochemical analysis of PrP in the infected mouse brain and analysis of the PrP^{res} molecular subtypes present.
2. To further extend the analysis of the vCJD agent strain by similar analysis after subsequent mouse-to-mouse passage.

5.2 Introduction

Variant CJD was first identified as a new form of human TSE in 1996 on the basis of a series of ten patients with a clinico-pathological phenotype that was distinct from other previously recognised forms of human TSE (Will et al., 1996). In contrast to the phenotypic heterogeneity observed in sCJD, these patients all showed a remarkably uniform clinical presentation, beginning with neuropsychiatric symptoms, such as anxiety or depression, dysaesthesia and ataxia. In addition, the neuropathological features were remarkably similar between cases, with the appearance of the distinctive “florid plaques” (Ironside et al., 1996). Subsequent biochemical analysis of brain tissue by Western blotting demonstrated a single PrP^{res} type associated with vCJD cases, characterised by a glycoform profile dominated by the diglycosylated fragment of PrP^{res} (Collinge et al., 1996; Head et al., 2004a). Crucially, the PrP^{res} type found in vCJD cases differed from that of the two major PrP^{res} types (type 1 and type 2A) associated with sCJD cases (Parchi et al., 1996). The prion hypothesis proposes that strain-specific information is enciphered in the different biochemical conformations of PrP. In accordance with this hypothesis, the unique PrP^{res} type associated with vCJD cases was used to argue that vCJD represents the result of infection with a single strain of agent which is distinct from other human TSEs.

The identification of vCJD so soon after the BSE epidemic in the UK raised concerns that vCJD was causally linked to the BSE agent. Now, almost twenty years on from the peak of the BSE epidemic, a wealth of experimental data supports the initial hypothesis that vCJD emerged as a result of human infection with the BSE agent, most probably through the consumption of contaminated meat products (Collinge et al., 1996; Lasmezas et al., 1996; Bruce et al., 1997; Hill et al., 1997; Scott et al., 1999).

As of December 2011, 176 cases of vCJD have been identified in the UK (<http://www.cjd.ed.ac.uk/figures.htm>). Although case numbers remain relatively low, having reached a peak incidence in 2000, a number of important concerns still surround vCJD. To date, all pathologically and biochemically confirmed clinical cases of vCJD for which codon 129 is known have occurred in patients who are homozygous for methionine at codon 129 of *PRNP*. The susceptibility of the other codon 129 subgroups to infection with the BSE agent and the development of clinical vCJD remain uncertain, although it appears that MV and VV individuals may also be susceptible to infection. In a retrospective study investigating the prevalence of vCJD in approximately 13,000 tonsil and appendix specimens, three specimens stained positive for the disease-associated prion protein (Hilton et al., 2004a). Further investigations confirmed two of these specimens were homozygous for valine at *PRNP* codon 129 (Ironsides et al., 2006). This was the first indication that the VV subgroup was susceptible to vCJD infection. In addition, the recent observations that vCJD appears to be transmissible by blood transfusion and by blood components highlighted two cases of peripheral infection in patients heterozygous at codon 129 (Peden et al., 2004; Peden et al., 2010). More recently, a case of clinical vCJD was also reported in a heterozygous individual (Kaski et al., 2009). However, as an autopsy was not carried out on this patient, the diagnosis was based on clinical criteria and has not been confirmed by neuropathological examination or by Western blot analysis of PrP^{res} type. These cases and the retrospective tonsil/appendix study suggest that all *PRNP* codon 129 subgroups may be susceptible to vCJD infection. This has been supported by animal models in which experimental transmission of vCJD in transgenic mice expressing human PrP with the codon 129 MM, MV and

VV genotypes showed all genetic groups may be susceptible to vCJD but that patients with the MV and VV genotype may have a lengthy preclinical disease (Bishop et al., 2006). The *PRNP* codon 129 genotype has been shown to influence the phenotype of other human TSEs (Parchi et al., 1999) and it is not known if any future cases of BSE transmission in these other genetic subtypes may show an altered clinical and pathological phenotype to that of vCJD in methionine homozygous individuals. The continued surveillance of vCJD and characterisation of the agent strain in these other genetic subgroups will play an important role in the identification of these cases.

The detection of infectivity and PrP deposition in peripheral tissues in vCJD (Bruce et al., 2001; Glatzel et al., 2003; Haik et al., 2003; Peden et al., 2004; Head et al., 2004b; Peden et al., 2006; Peden et al., 2007; Notari et al., 2010) and now the apparent transmission of vCJD via infectivity in blood and blood products (Llewelyn et al., 2004; Peden et al., 2004; Wroe et al., 2006; Health Protection Agency, 2009b; Peden et al., 2010), highlight the risk of possible secondary transmission of vCJD. An additional concern is the theoretical possibility of alterations in agent strain on secondary transmission, which may result in a modified disease phenotype and biochemical profile.

Experimental strain typing in mice and biochemical characterisation of PrP^{res} has produced convincing evidence that vCJD is caused by the same strain of agent that caused BSE (Collinge et al., 1996; Bruce et al., 1997; Hill et al., 1997). However the existence of more than one BSE strain, which may have already infected humans, has been suggested in a publication examining the transmission of BSE into transgenic mice expressing human PrP with methionine at codon 129 (Asante et al., 2002). In this study, Western blot analysis of the PrP^{res} type in the mouse brain after challenge with BSE resulted in more than one biochemical phenotype, one with a BSE/vCJD-like profile and a second that was indistinguishable from that of sCJD. This raises concerns that patients with a phenotype consistent with sCJD may have actually acquired the disease from infection with the BSE agent. A similar finding has been reported in a different humanised transgenic mouse line expressing methionine

homozygosity following inoculation with vCJD (Beringue et al., 2008). However, in this study the vCJD/BSE biochemical profile was retained in the spleen.

In response to these and other concerns surrounding the vCJD agent, this chapter aims to provide a full characterisation of the vCJD agent on primary transmission in panels of wild-type mice. Extensive studies of the transmission of vCJD in numerous transgenic mouse models have provided valuable information on the vCJD agent. However, at present the characterisation of different TSE strains is most highly developed using transmission into non-transgenic (wild-type) mice. The original vCJD strain typing paper by Bruce and co-workers (Bruce et al., 1997) remains one of the most compelling pieces of evidence that vCJD resulted from the same major strain of agent that caused BSE and that the BSE/vCJD agent is distinct from the causing sCJD. A limitation to this paper is that the characterisation of the vCJD agent was based on the incubation period and lesion profiles produced on primary transmission from three vCJD cases to a single wild-type mouse line (RIII). The data presented in this chapter significantly extends these studies by increasing the number of cases transmitted to ten and providing the complete incubation period and lesion profile data in a larger panel of mouse lines. In addition the analysis is extended to include the biochemical and immunohistochemical analysis of the abnormal prion protein accumulation in the mice. During the transmission of different TSE isolates to mice, different strains are often isolated from a single source by serial passage through mice of different *Prn-p* genotypes, so for a full characterisation of vCJD it was necessary to do strain typing both on primary transmission to mice and on subsequent mouse-to-mouse passage of the isolate.

5.3 Experimental results: Primary transmissions

Eleven primary transmission experiments were carried out in panels of wild-type mice from brain homogenate prepared from ten neuropathologically confirmed cases of vCJD (two separate transmission experiments were carried out with different frontal cortex samples taken from one of the vCJD patient). Patient details for these ten cases (CJD 28 – CJD 37) are provided in Chapter 2, Table 2.1. Brain homogenate was prepared from either grey matter tissue from the cerebellar cortex or the frontal cortex, with inoculations either via a single IC injection or a combination of the IC and IP routes (Chapter 2, Table 2.3).

5.3.1 Incubation periods and incidence of disease

All eleven vCJD brain homogenates transmitted successfully to all three mouse lines (RIII, C57BL and VM) and the C57BL x VM cross, with the appearance of a progressive clinical disease and/or the presence of TSE-specific vacuolar degeneration in the brains of mice after histological analysis. The incidence of clinical disease and vacuolar pathology in each experimental group, with results grouped according to brain region inoculated and route of injection are detailed in Table 5.1. Mean incubation periods were calculated for experimental groups with a minimum of five mice receiving a positive score for both clinical signs of disease and the presence of vacuolar pathology in the brain. Mean incubation period data for all eleven primary transmissions are presented in Table 5.1 and Figure 5.1.

Table 5.1: Primary transmission from vCJD brain homogenate

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice challenged with brain homogenate from vCJD cases. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Incubation periods in days are shown in brackets \pm S.E.M.

	Mouse line							
	RIII		C57BL		VM		C57BL x VM	
Source	Clin	Vac path	Clin	Vac Path	Clin	Vac path	Clin	Vac path
vCJD 28*	14/14 (307 \pm 4)	14/14	10/10 (428 \pm 4)	10/10	13/13 (464 \pm 7)	13/13	6/9 (673 \pm 10)	9/9
vCJD 29*	17/18 (309 \pm 8)	18/18	15/16 (422 \pm 8)	15/16	16/21 (492 \pm 12)	16/21	11/14 (699 \pm 3)	14/14
vCJD 30*	17/17 (306 \pm 3)	16/17	13/15 (426 \pm 9)	15/15	22/22 (444 \pm 6)	21/22	14/19 (685 \pm 6)	19/19
vCJD 31†	13/20 (402 \pm 4)	16/20	11/16 (522 \pm 19)	11/16	7/22 (551 \pm 17)	17/22	4/22 (728,728,742,742)	19/22
vCJD 32†	17/25 (383 \pm 7)	24/25	9/18 (524 \pm 8)	12/18	8/23 (586 \pm 12)	20/23	2/24 (783,621)	16/24
vCJD 33†	20/23 (362 \pm 8)	20/23	19/21 (479 \pm 9)	17/21	11/19 (571 \pm 6)	15/19	3/19 (686,750,750))	15/19
vCJD 34†	21/22 (342 \pm 6)	22/22	12/17 (466 \pm 16)	16/17	15/20 (531 \pm 14)	19/20	6/22 (650 \pm 38)	19/22
vCJD 31‡	13/13 (359 \pm 10)	10/13	nd	nd	9/13 (556 \pm 13)	12/13	nd	nd
vCJD 35‡	17/18 (353 \pm 9)	17/18	nd	nd	15/22 (510 \pm 11)	20/22	nd	nd
vCJD 36‡	18/20 (374 \pm 10)	17/20	nd	nd	21/22 (523 \pm 12)	21/22	nd	nd
vCJD 37‡	17/21 (382 \pm 6)	16/21	nd	nd	11/18 (536 \pm 9)	16/18	nd	nd

* Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from vCJD cerebellar tissue

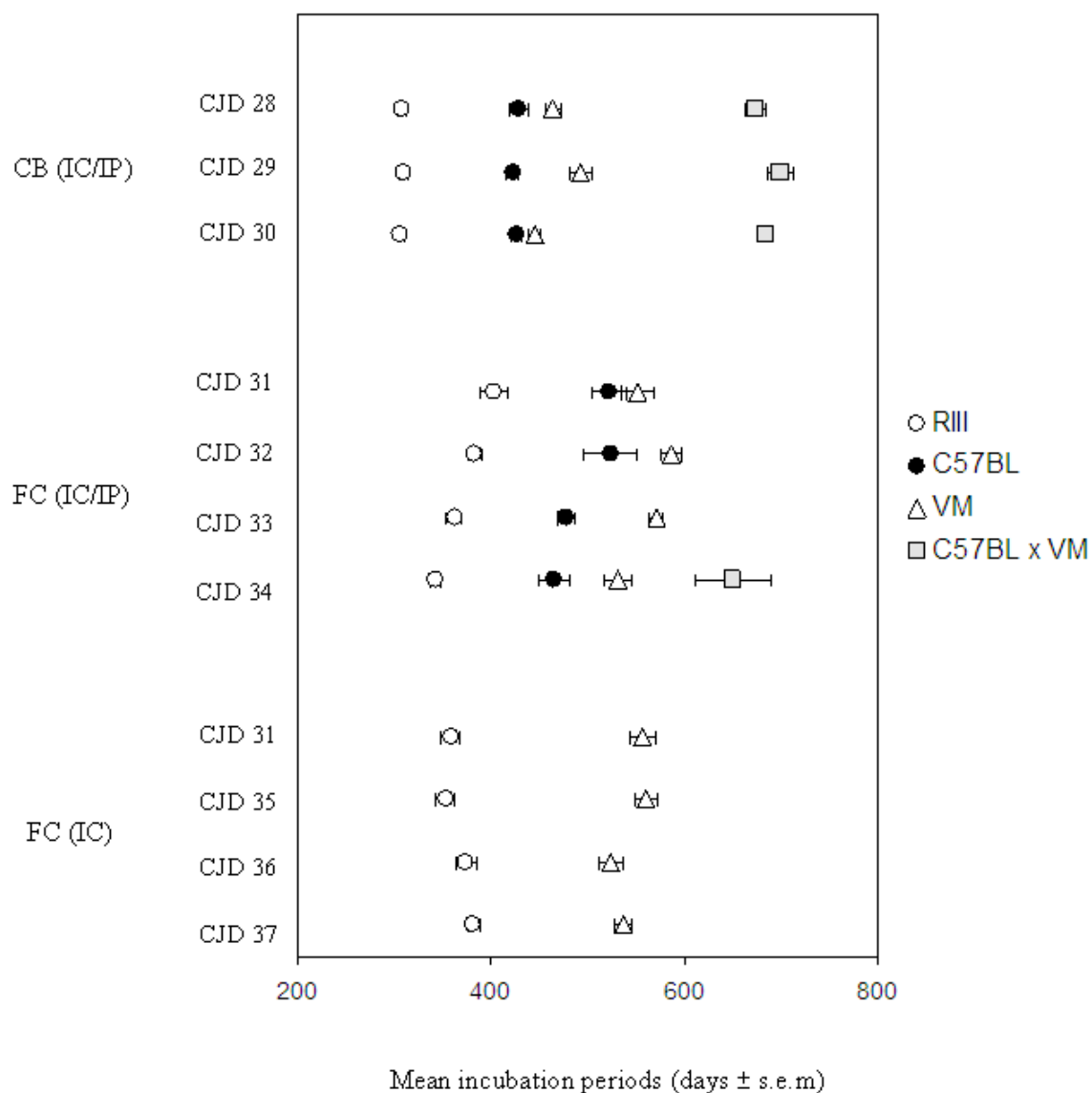
† Intracerebral and intraperitoneal injection with 10% brain inoculum prepared from vCJD frontal cortex tissue

‡ Intracerebral injection with 10% brain inoculum prepared from vCJD frontal cortex tissue

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Figure 5.1: Incubation times in vCJD challenged mice

Mean incubation periods in RIII, C57BL, VM and C57BL x VM mice after experimental challenge with brain homogenate from ten vCJD cases. Data are mean \pm S.E.M. (FC) frontal cortex, (CB) cerebellar cortex, (IC) intracerebral inoculation (IP) intraperitoneal inoculation.



Each vCJD brain homogenate gave the same order of appearance of clinical signs in the mouse lines inoculated. The shortest incubation periods were consistently observed in the two *Prn-p^a* mouse lines (RIII and C57BL) with the longest in the C57BL x VM cross. The RIII and C57BL mice, although of the same *Prn-p* genotype, consistently showed incubation period differences of around 100 days, with mean incubation periods up to a standard clinical endpoint ranging from 306 ± 3 to 402 ± 4 days (\pm S.E.M) in the RIII mouse line compared to 422 ± 8 to 524 ± 1 days (\pm S.E.M) in the C57BL line. Incubation periods were broadly similar irrespective of the brain region (frontal cortex or cerebellum) inoculated or the route of injection. However, the exact timing of the appearance of clinical symptoms showed some variability between individual brain homogenates, most noticeably with homogenate prepared from the frontal cortex of the brain. Shorter incubation periods were observed with homogenate prepared from cerebellar cortex, independent of the route of injection. In RIII mice, inoculation via a combination of the IC and IP routes with homogenate prepared from frontal cortex produced means of between 342 ± 6 and 402 ± 4 days (\pm S.E.M.) compared to means of between 306 ± 3 and 309 ± 8 days (\pm S.E.M.) with homogenate from the cerebellar hemisphere inoculated via the same route. These results imply that levels of infectivity may be higher in the cerebellum region than in the frontal cortex.

5.3.2 Patterns of vacuolar pathology- lesion profiles

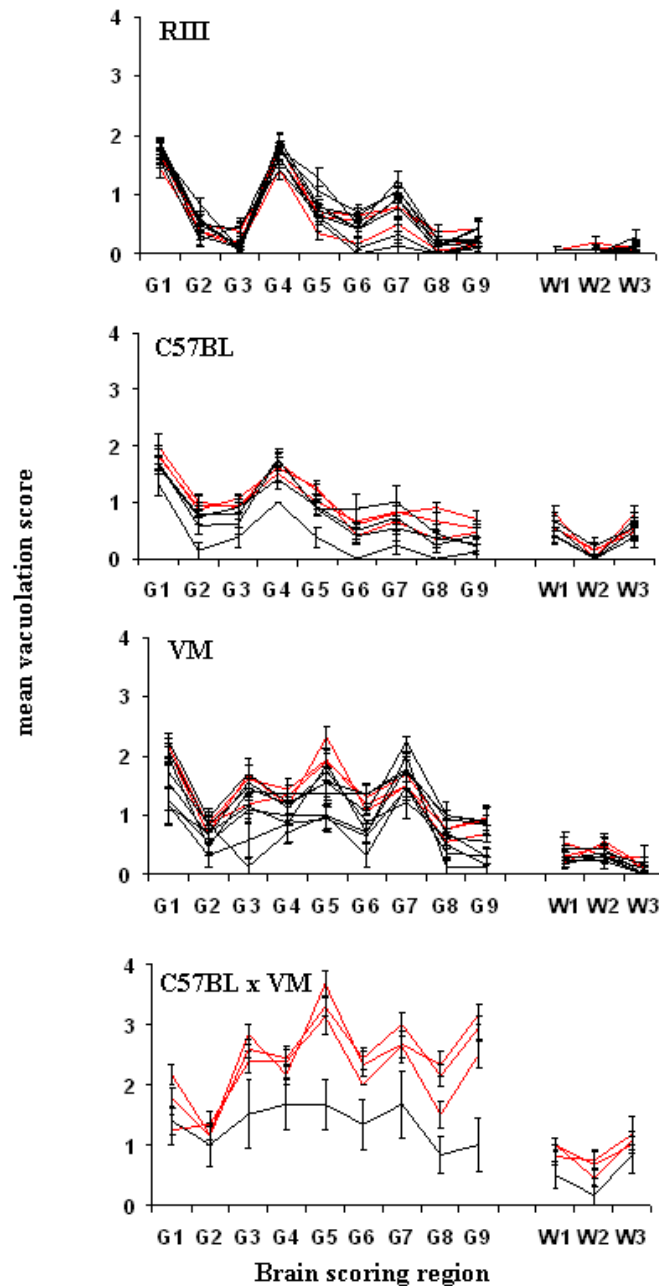
Vacuolar pathology typical of TSE infection was observed in the majority of mice challenged with each vCJD brain homogenate (601/678 mice analysed) (Table 5.1). This confirmed the diagnosis of a TSE for all clinically affected mice. All but three experimental groups (C57BL x VM mice inoculated with brain homogenate from patients CJD 31, CJD 32 and CJD 33) had sufficient mice with a positive score for clinical signs of disease and vacuolar degeneration in the brain for the construction of a lesion profile. The lesion profiles for all three mouse lines and the C57BL x VM cross following inoculation with brain homogenate from all ten vCJD cases are shown in Figure 5.2. A remarkably similar pattern of mild-to-moderate vacuolation was observed with each vCJD homogenate regardless of the brain region used in the inoculation or the route of injection. There was some variability in the intensity of the vacuolation between individual homogenates, most noticeably in the VM and

C57BL x VM cross. In the C57BL x VM mice, the profile was much lower in the one group inoculated with frontal cortex tissue compared to the profiles of the three groups inoculated with cerebellar tissue. This was also found to a lesser extent in VM mice, where profiles were also slightly higher in those groups inoculated with cerebellar tissue. However, in general the pattern of vacuolation was similar.

In contrast to transmission of sCJD from brain homogenates (Chapter 4), patterns of vacuolar degeneration in vCJD-inoculated mice differed between the different mouse *Prn-p* genotypes. The RIII and C57BL mice, both of the *Prn-p^a* genotype, produced a distinct pattern of mild-to-moderate vacuolation, consistently targeting the hypothalamus (scoring region G4) with some prominent vacuolation in the medulla (scoring region G1) and the septum (scoring region G7). This pattern differed from those in the VM (*Prn-p^b*) mice and the C57BL x VM cross which show a more widespread targeting of moderate vacuolation throughout the scoring regions of the mouse brain. In general, vacuolar pathology was more severe in the C57BL x VM cross, perhaps reflecting the longer incubation period in this mouse line. These vacuolation profiles clearly show a similarity in lesion distribution between the ten vCJD cases.

Figure 5.2: Lesion profiles for vCJD inoculated mice

Lesion profiles are constructed for groups in which there was a minimum of five mice with a positive score for both clinical signs of TSE and TSE-specific vacuolar pathology. Vacuolation was scored at nine specified grey (G) matter regions and three white (W) matter regions of the mouse brain. Lesion profiles are shown with standard error bars (\pm S.E.M). Each line represents a different vCJD transmission. (Black line) brain homogenate prepared from frontal cortex tissue; (red line) brain homogenate prepared from the cerebellar cortex. No distinction is made between different inoculation routes



5.3.3 Deposition of PrP in the mouse brain

Lesion profiles in vCJD-infected mice differed between mouse lines of different PrP genotypes. Therefore, in investigating the patterns of PrP deposition in the brain of vCJD-infected mice, all four mouse lines were analysed. Immunohistochemistry was carried out on 5µm formalin fixed, formic acid treated paraffin sections of brain from mice challenged with five different vCJD sources. In each experiment, seven mice of each mouse line were analysed; five inoculated with CJD brain homogenate and two saline injected controls. All vCJD-infected mice selected for analysis had received a positive score for both clinical disease and vacuolar pathology. Immunohistochemistry was carried using the highly sensitive Catalyzed Signal Amplification System (CSA) as described in chapter 2, section 2.6.4.

Deposition of the abnormal prion protein was observed in all vCJD challenged mice examined, further confirming the successful transmission of a TSE from vCJD brain homogenate. Patterns of PrP accumulation in the mouse brain were remarkably similar between mice inoculated with the different vCJD homogenate. Overall, immunohistochemistry showed an intense, widespread deposition of granular and peri-cellular PrP staining throughout the brains of infected mice at clinical endpoint. Representative examples of brain sections, from each mouse line, immunolabelled for PrP using the anti-PrP antibody 6H4 are shown in Figure 5.3. In contrast to lesion profile data, patterns of PrP deposition were similar in the different mouse PrP genotypes, with the most prominent depositions of PrP in the medial habenular nucleus (MHb), the medulla, the thalamus and around areas of intense vacuolation (Figure 5.3). Granular PrP deposits were also observed in the molecular and granular layer of the cerebellum. Subtle differences in PrP staining patterns were observed in the hippocampal region between the different *Prn-p* genotypes; *Prn-p^a* mice (RIII and C57BL) showed a faint granular deposition of PrP within the CA2 region of the hippocampus (Figure 5.3b and d) while the *Prn-p^b* (VM) and the C57BL x VM cross showed a more intense deposition of PrP targeting the dentate gyrus (Figure 5.3f and h). Amyloid plaques, a common and characteristic feature of vCJD, were not a prominent feature in the mice, and were observed only in the VM mice and the C57BL x VM mice, mainly restricted to the corpus callosum (Figure 5.4a and b).

Intensity of PrP staining was consistently higher in the *Prn-p^b* mice and the C57BL x VM cross when compared to the *Prn-p^a* mice, perhaps a reflection of the longer incubation periods occurring in these two mouse *Prn-p* genotypes. No evidence of PrP accumulation was observed in any of the saline injected mice analysed (data not shown).

Figure 5.3: PrP deposition in vCJD inoculated mice

The deposition of PrP in the mice was examined at five defined brain levels of which two are shown here (**a, c, e, f**) the medulla and cerebellum, (**b, d, f, h**), hippocampus, habenula, thalamus, corpus callosum and cerebral cortex. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Corpus callosum (CC), CA2 region of the hippocampus (CA2), dentate gyrus (DG), medial habenular nucleus (MHb), thalamus (Th). Original magnification; x25 (a, c, e, g), x50 (b, d, f, h).

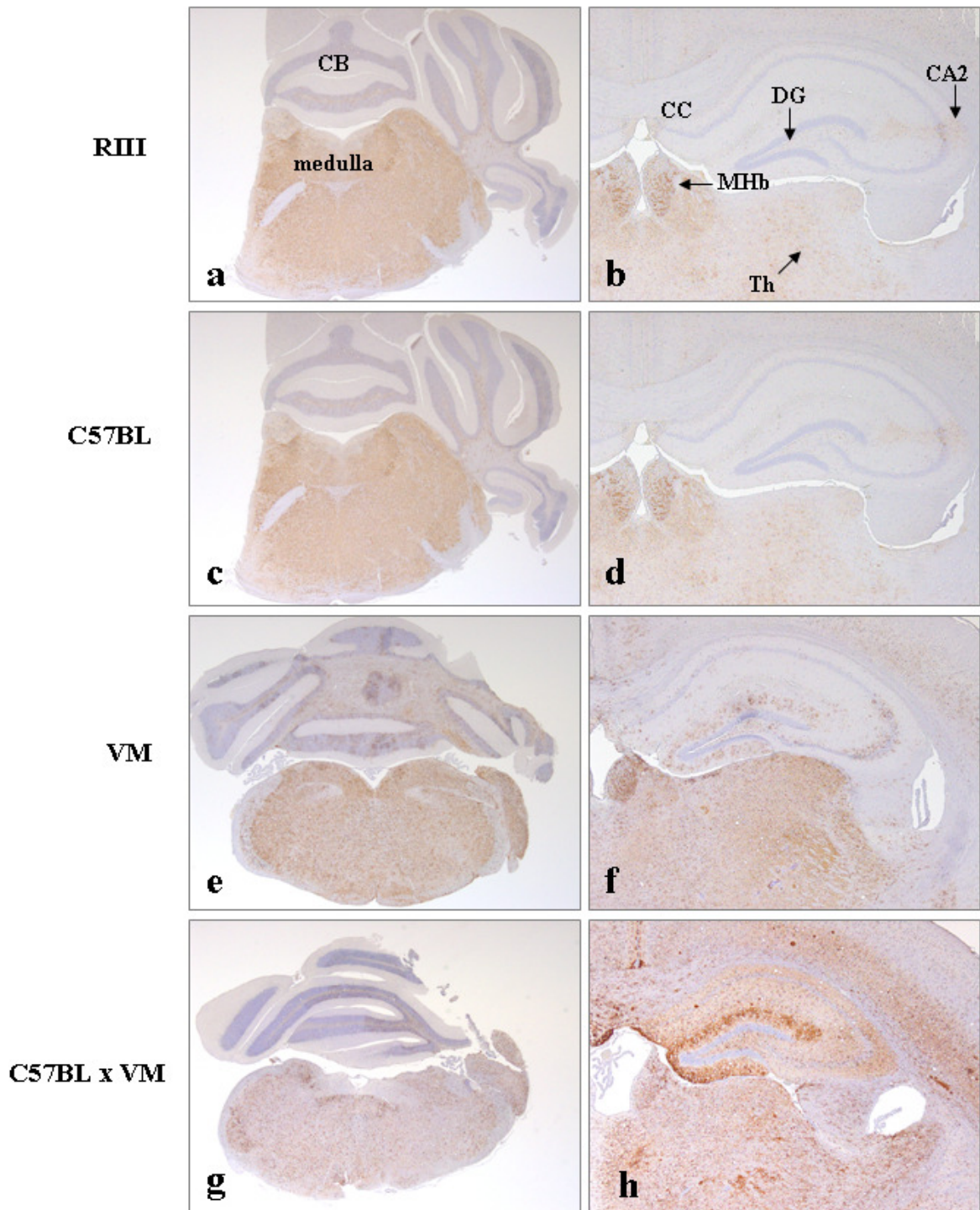
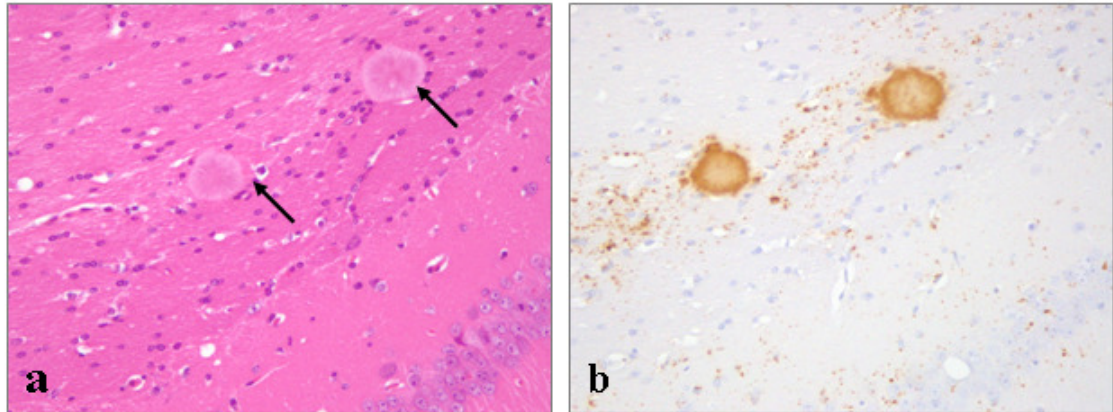


Figure 5.4: Amyloid plaques in vCJD inoculated mice

Amyloid plaques in a VM mouse challenged with vCJD brain homogenate. **(a)** Haematoxylin and eosin staining showing the presence of amyloid plaques (arrows) within the corpus callosum. **(b)** Serial section labelled with the monoclonal anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification x400.

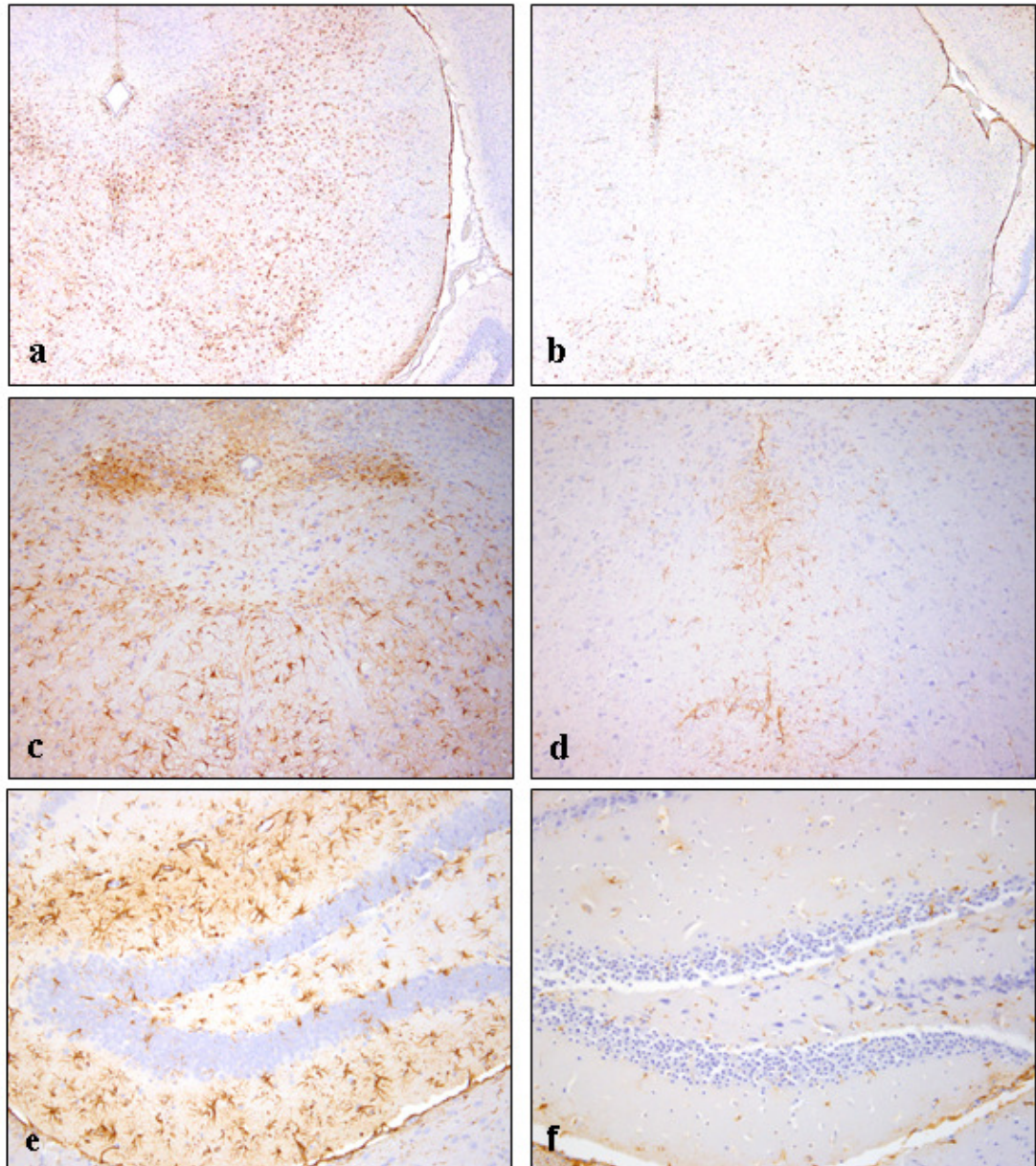


5.3.4 Astrocytic proliferation in vCJD challenged mice

Astrocytosis within the brain is a characteristic feature of TSEs. Immunolabeling for GFAP was carried out in the mice to determine if there was an upregulation in GFAP in the mouse brain that would indicate the development of astrocytosis. All mice examined showed a widespread and marked upregulation in GFAP staining when compared to saline injected controls, indicative of astrocytosis within vCJD-infected mice (Figure 5.5). This upregulation in GFAP was observed in mice of all PrP genotypes examined. Staining for GFAP was most extensive around areas of PrP accumulation; specifically in the medulla, thalamus and hippocampus region and throughout the mid-brain. As was observed with the deposition of PrP, astrocytosis appeared more extensive in the *Prn-p^b* mice and *Prn-P^{ab}* cross when compared to the *Prn-p^a* mice. This is most likely due to the longer incubation periods in these mice.

Figure 5.5: GFAP labelling in vCJD inoculated mice

Extensive astrocytosis within the brains of vCJD challenged mice (**a, c, e**) when compared to saline injected controls (**b, d, f**). GFAP staining was most extensive within the (**a**) midbrain and the (**c**) medulla of infected mice compared to (**b, d**) saline inoculated controls. The dentate gyrus showing an upregulation of GFAP in (**e**) infected mice when compared to (**f**) uninfected controls. All sections shown are from VM mice and are labelled with the anti-GFAP antibody in combination with the ABC detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x50 (a, b), x100 (c, d), x200 (e, f).



5.3.5 Biochemical isotyping of PK resistant PrP^{Sc}

To investigate whether the vCJD biochemical phenotype is maintained on transmission to mice, Western blot analysis of the PrP^{res} type was carried out on vCJD-infected mice from all mouse PrP genotypes using standard Western blotting methods as described in Chapter 2, section 2.7. All mice examined were scored positive for the appearance of clinical signs of disease and vacuolar pathology.

PrP^{res} was readily detected in the brain in all vCJD-infected mice examined. Results showed that important aspects of the biochemical phenotype of vCJD brain PrP^{res} were maintained after primary transmission to the mice. Western blot analysis of PrP^{res} in CNS tissue from infected mice of all four mouse lines produced a glycosylation pattern closely resembling that of vCJD brain tissue, characterised by the predominance in the diglycosylated form of the protein (Figure 5.6). A subtle difference in the electrophoretic mobility of the unglycosylated form of the protein was found between the mouse and human brain samples, suggesting some variation in the size of the protease-resistant core of the mouse and human protein, either because of differences in the exact truncation point for PK or because of sequence differences between human and murine PrP. A feature of the experimentally infected mice, not observed in vCJD in humans, was the appearance of the unglycosylated form of the protein as a doublet composed of distinct upper and lower bands (Figure 5.7). The relative amounts of the two bands in the doublet varied and appeared to correlate with the different *Prn-p* genotypes; the *Prn-p^a* mice appeared to show predominance of the lower band of the doublet, whereas *Prn-p^b* mice had predominance in the upper band. Both the upper and lower band of the doublet was readily discernable in the C57BL x VM cross.

Figure 5.6: Western blot analysis of PrP^{res} in vCJD inoculated mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after experimental transmission of vCJD brain homogenate. Tissues are analysed after digestion with proteinase K and probed with the anti-PrP antibody 6H4. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).

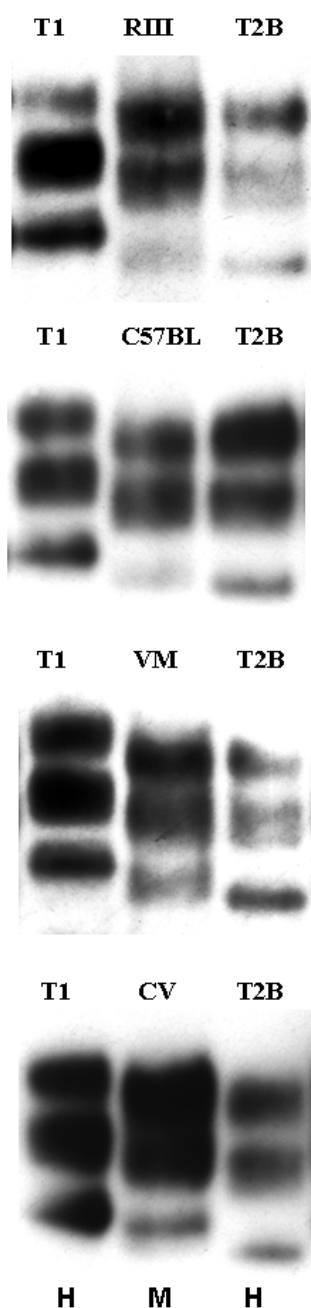
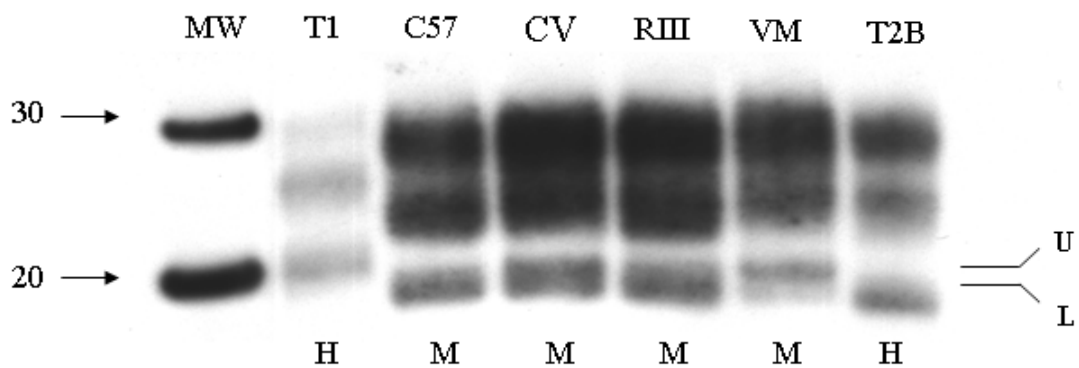


Figure 5.7: Further Western blot analysis of PrP^{res} in vCJD inoculated mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL (C57), VM and C57BL x VM (CV) mice after experimental transmission of vCJD brain homogenate. Tissues are analysed after digestion with proteinase K and probed with the anti-PrP antibody 6H4. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 diagnostic standard (T1) and a human vCJD diagnostic standard (T2B). Molecular weight makers (MW) are shown in kDa. (U) Upper band of the unglycosylated fragment, (L) Lower band of the unglycosylated fragment.



5.4 Experimental results: mouse-to-mouse passage

Further characterisation of the agent strain in vCJD was carried out by conducting analysis of two serial mouse-to-mouse passages of brain isolates derived from two different vCJD cerebellar samples (patients CJD 28 and CJD 29). Homogenates were prepared from the brains of a single RIII, VM or C57BL mouse which had shown both clinical and pathological signs of a TSE after primary challenge of vCJD. Homogenates were inoculated via a single IC injection into all three mouse lines and the C57BL x VM cross. In each case, this was repeated for a further mouse-to-mouse passage. A diagrammatic representation of the sub-passage experiments is shown in chapter 4, Figure 4.24.

5.4.1 Incubation periods and incidence of disease

Each brain isolate transmitted successfully in mice of all *Prn-p* genotypes. Details of the incidence of clinical disease and vacuolar degeneration after passage in *Prn-p^a* and *Prn-p^b* mice are provided in Tables 5.2a and 5.2b, respectively. The two cerebellar sources provided similar results, with the incidence of clinical and pathological disease 100% in most groups and close to 100% in the others. Mean incubation periods shortened dramatically on the first mouse-to-mouse passage for all experimental groups, consistent with the removal of the ‘species barrier’ between the donor and recipient which was present in the primary transmission (Figure 5.8). Incubation periods were broadly similar between the first and second mouse-to-mouse passages, suggesting that the vCJD agent stabilises almost immediately on passage in mice. Sub-passage in *Prn-p^a* mice (RIII or C57BL) resulted in an identical ranking of incubation periods in the mouse genotypes to that seen in primary transmissions (Figure 5.8a and b), whereas a change in order was found on sub-passage in *Prn-p^b* mice (VM) (Figure 5.8c). Furthermore, sub-passage in *Prn-p^b* mice produced the shortest incubation period observed in this transmission series, with mean incubation periods as low as 106 days in VM recipients (Figure 5.8c). These distinct rankings of incubation period indicate that serial mouse passage of these two vCJD brain isolates has resulted in the isolation of two variant strains of agent, selected according to which has the shorter incubation period in the *Prn-p* genotype of host used for passage.

Table 5.2a: Serial passage of vCJD brain isolates in *Prn-p^a* mice

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice after two serial mouse-to-mouse passages of isolates derived from cerebellar homogenates from two vCJD patients. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Incubation periods in days are shown in brackets (mean \pm S.E.M. for groups).

	Recipient mouse lines							
	RIII		C57BL		VM		C57BL x VM	
Donor tissue source	Clin	Vac path	Clin	Vac path	Clin	Vac path	Clin	Vac path
vCJD 28 RIII 1 st pass	12/12 (180 \pm 1)	12/12	8/9 (208 \pm 3)	9/9	11/11 (365 \pm 6)	11/11	10/10 (560 \pm 7)	10/10
2 nd pass	9/10 (171 \pm 1)	10/10	12/12(194 \pm 3)	12/12	10/12 (326 \pm 7)	12/12	10/10 (527 \pm 2)	10/10
C57BL 1 st pass	10/10 (169 \pm 2)	10/10	4/4 (199 \pm 2)	4/4	10/11 (336 \pm 9)	11/11	10/10 (548 \pm 4)	10/10
2 nd pass	9/9 (165 \pm 2)	9/9	8/9 (199 \pm 2)	9/9	12/12 (332 \pm 9)	12/12	10/10 (546 \pm 13)	10/10
vCJD 29 RIII 1 st pass	10/10 (175 \pm 1)	10/10	12/12(204 \pm 2)	12/12	12/12 (316 \pm 8)	12/12	9/10(547 \pm 4)	10/10
2 nd pass	12/12 (164 \pm 1)	12/12	12/12(198 \pm 1)	12/12	11/11 (304 \pm 10)	11/11	9/9 (527 \pm 5)	9/9
C57BL 1 st pass	12/12 (176 \pm 3)	12/12	11/11(208 \pm 1)	11/11	11/11 (341 \pm 9)	11/11	8/9 (546 \pm 7)	9/9
2 nd pass	11/11 (164 \pm 1)	11/11	10/10(200 \pm 2)	10/10	11/12(297 \pm 20)	12/12	8/9 (523 \pm 8)	9/9

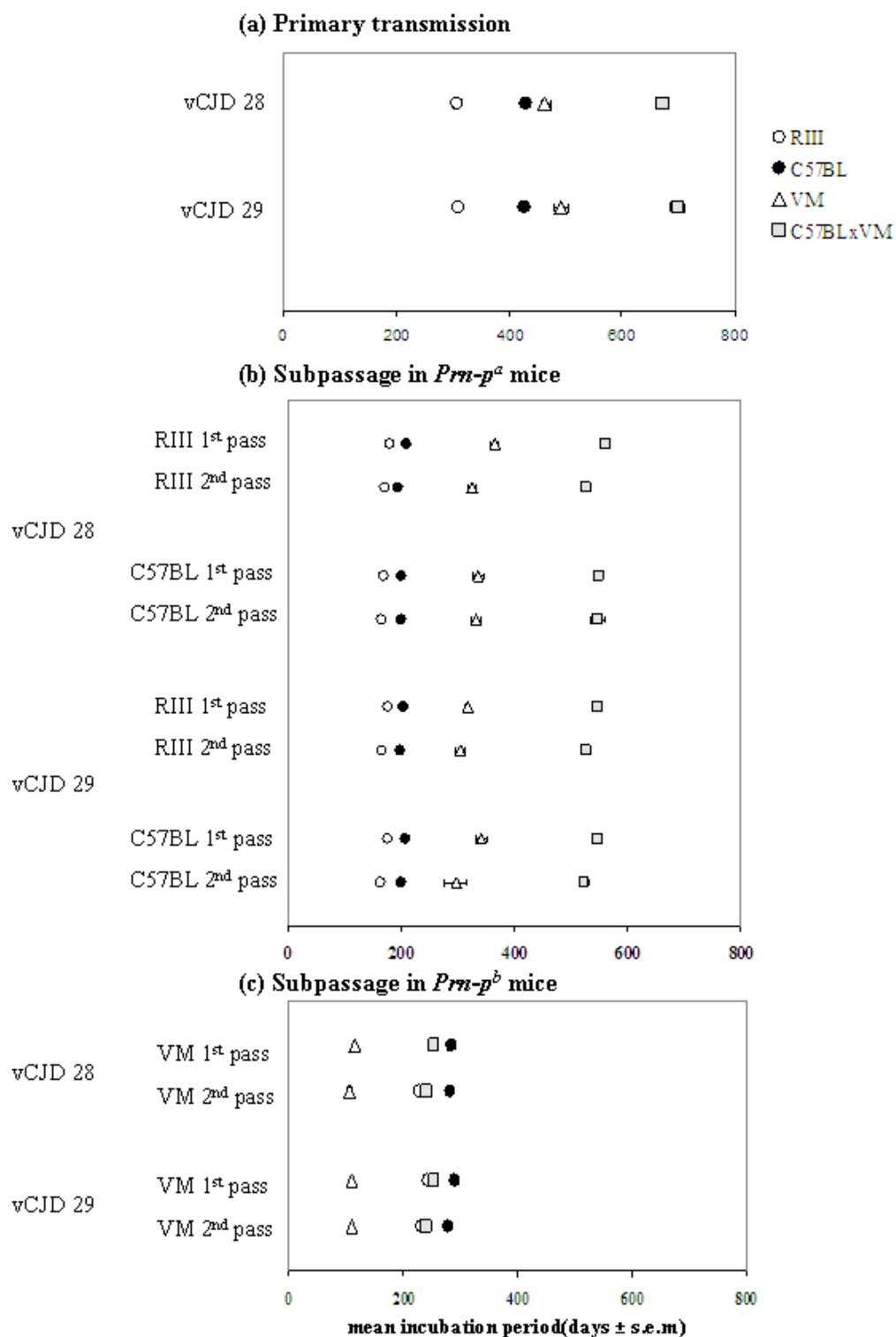
Table 5.2b: Serial passage of vCJD brain isolates in *Prn-p^b* mice

Incidence of clinical signs (Clin) and vacuolar degeneration (Vac path) in mice after two serial mouse-to-mouse passages of isolates derived from cerebellar tissue from two vCJD patients. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Incubation periods in days are shown in brackets (mean \pm S.E.M. for groups).

	Recipient mouse lines							
	RIII		C57BL		VM		C57BL x VM	
Donor tissue source	Clin	Vac path	Clin	Vac path	Clin	Vac path	Clin	Vac path
vCJD 28 VM 1 st pass	10/10 (251 \pm 1)	10/10	8/8 (285 \pm 6)	8/8	12/12 (116 \pm 1)	12/12	10/10 (255 \pm 1)	10/10
2 nd pass	7/7 (228 \pm 1)	7/7	7/8 (282 \pm 1)	7/8	11/11 (106 \pm 3)	11/11	9/9 (241 \pm 2)	9/9
vCJD 29 VM 1 st pass	8/9 (242 \pm 7)	9/9	6/9 (290 \pm 7)	9/9	11/11 (110 \pm 1)	11/11	9/9 (255 \pm 1)	9/9
2 nd pass	8/9 (231 \pm 4)	9/9	7/7 (278 \pm 7)	7/7	10/10 (110 \pm 2)	10/10	9/10 (240 \pm 1)	10/10

Figure 5.8: Incubation times in mice after sub-passage of vCJD MM1

Mean incubation periods in RIII, C57BL, VM and C57BL x VM mice after (a) primary transmission of two vCJD cerebellar homogenates and following first and second mouse passage of the same cerebellar isolates in (b) *Prn-p^a* and (c) *Prn-p^b* mice. Data are mean \pm S.E.M

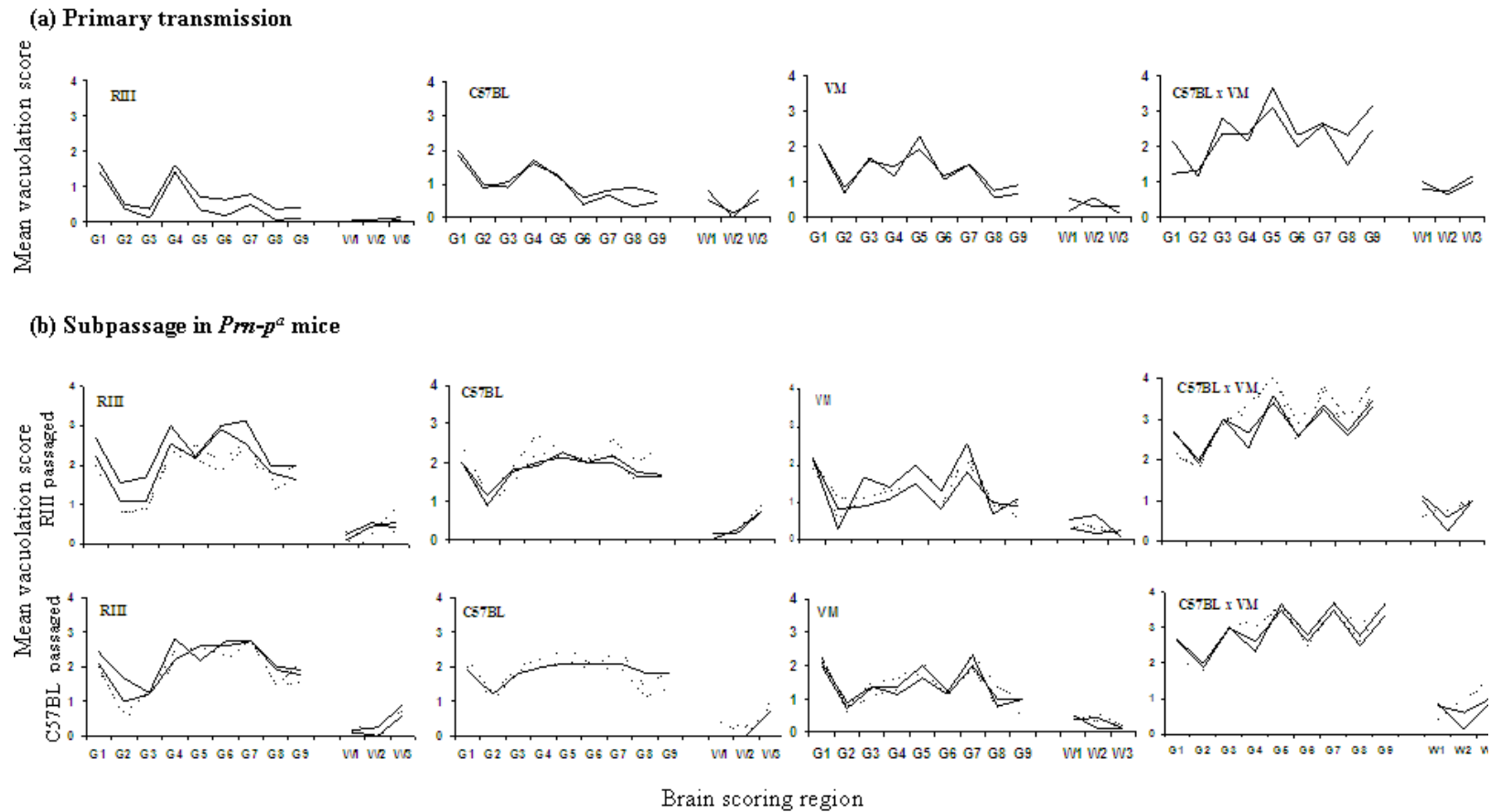


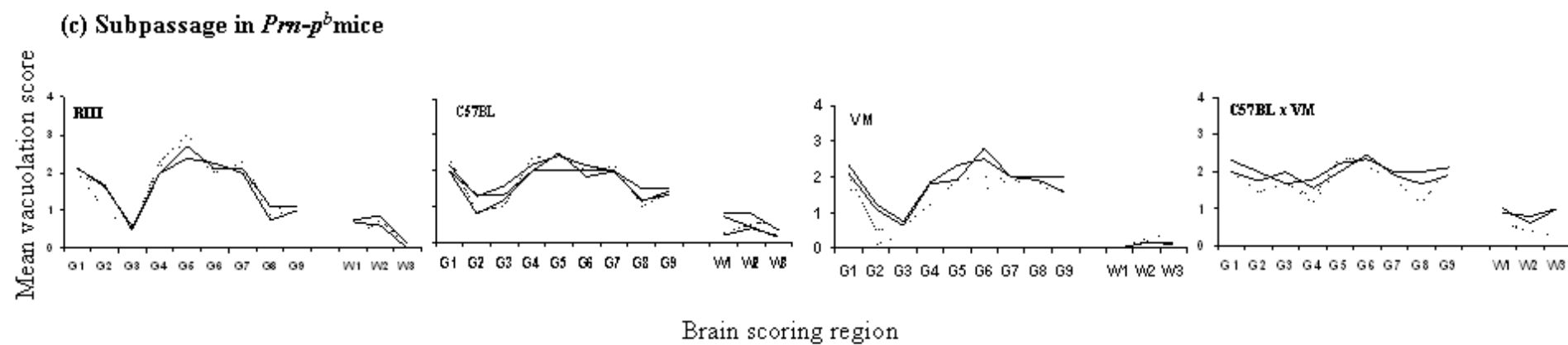
5.4.2 Patterns of vacuolar pathology- lesion profiles

Lesion profiles comparing the primary transmissions of vCJD from patients CJD 28 and CJD 29 with lesion profiles after subsequent serial passage of these isolates in different mouse *Prn-p* genotypes are presented in Figure 5.9. Both brain isolates showed a remarkably similar pattern of vacuolar pathology in the mouse brain indicating the same strain of agent within each brain isolate. Lesion profiles from the serial passages showed a modified pattern of vacuolar pathology when compared to the primary transmissions (Figure 5.9). In the RIII mouse line, vacuolar pathology was observably more severe after serial passage of brain isolates in both mouse genotypes when compared to the primary transmissions particularly in the thalamus (G5), hippocampus (G6) and septum (G7). Although incubation period data indicated the isolation of two different agent strains following passage in the different mouse *Prn-p* genotypes, this was not clearly reflected in the lesion profiles, except in the case of C57BL x VM mice, in which the VM-passaged profile showed a less severe vacuolar pathology in the brain. No significant differences were observed in the lesion profiles between first and second mouse passages, thus providing no evidence of strain selection or host modification beyond the primary transmission stage. As was observed in the primary passage of vCJD brain homogenate, slight, but consistent differences did occur between the different recipient mouse lines showing the influence that host genetic factors have on lesion profiles.

Figure 5.9: Lesion profiles for mice following sub-passage of vCJD

Lesion profiles are constructed from groups in which a minimum of five mice had clinical signs of a TSE and a positive score for TSE associated vacuolar pathology. Vacuolation was scored at nine specified grey (G) matter regions and three white (W) matter regions of the mouse brain. Lesion profiles shown are from mice following the (a) primary transmission from cerebellar tissue from two vCJD cases and subsequent passage in (b) *Prn-p^a* and (c) *Prn-p^b* mice. Lesion profiles are shown without standard error bars (\pm S.E.M). (Solid lines) represent first mouse-to-mouse passage, (Dashed lines) second mouse-to-mouse passage.





5.4.3 Deposition of PrP in the mouse brain

Patterns of PrP deposition in the mouse brain were investigated following the first and second mouse-to-mouse passage of both brain isolates. Immunohistochemistry was carried out on 5µm formalin fixed, formic acid treated, brain sections from all three recipient mouse lines (RIII, VM and C57BL) and the C57BL x VM cross using the anti-PrP antibody 6H4 in combination with the CSA detection method as previously described (Chapter 2, section 2.6.4). All mice examined had received a positive score for the presence of clinical disease and vacuolar pathology.

Results confirmed the deposition of PrP in the brain of all mice examined. Overall, immunohistochemistry showed a consistently more intense deposition of PrP on serial passage when compared to the primary transmissions (Figure 5.10). The general distribution of PrP deposition in the mice following sub-passage was similar to that found in the same mouse lines after primary transmission of vCJD, with a widespread and intense granular and peri-cellular deposition of PrP within the MHb, medulla, hippocampus and the thalamus (Figure 5.10). The subtle differences observed in the distribution of PrP within the hippocampus region of the mouse brain between the different *Prn-p* genotypes were conserved on serial passage. This was most obvious in the VM and C57BL x VM mouse lines with intense PrP accumulation targeting the dentate gyrus (Figure 5.10g-l). However, with a generally more intensive staining and distribution of PrP, the whole hippocampal region of the brain showed PrP pathology. In addition to the intense PrP positivity observed in the mice, there were numerous plaque-like deposits observed in the corpus callosum in *Prn-p^a* (RIII and C57BL) mice on serial passage in both mouse PrP genotypes (Figure 5.10b, c, e and f). Furthermore, in contrast to the primary transmissions in which amyloid plaques were observed only in VM mice and the C57BL x VM cross, all four mouse lines showed the presence of amyloid plaques following the serial passage of brain isolates in *Prn-p^a* and *Prn-p^b* mice. The distribution of these amyloid plaques was also more widespread than those in the primary transmissions, with plaques observed in the hippocampus, thalamus and in the cerebral cortex (Figure 5.11). No significant differences in patterns of PrP accumulation were

observed between first and second passage (data not shown) or between $Prn-p^a$ passaged and $Prn-p^b$ passaged isolates (Figure 5.10).

Figure 5.10: PrP deposition after serial passage of vCJD brain isolates

Deposition of PrP in wild-type mice following the primary transmission of vCJD brain homogenate (**a, d, g, j**) and after subsequent first serial passage of vCJD brain isolates in *Prn-p^a* (**b, e, h, k**) and *Prn-p^b* (**c, f, i, l**) mice. Brain regions shown are hippocampus, medial habenular nucleus, thalamus and corpus callosum. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification x50.

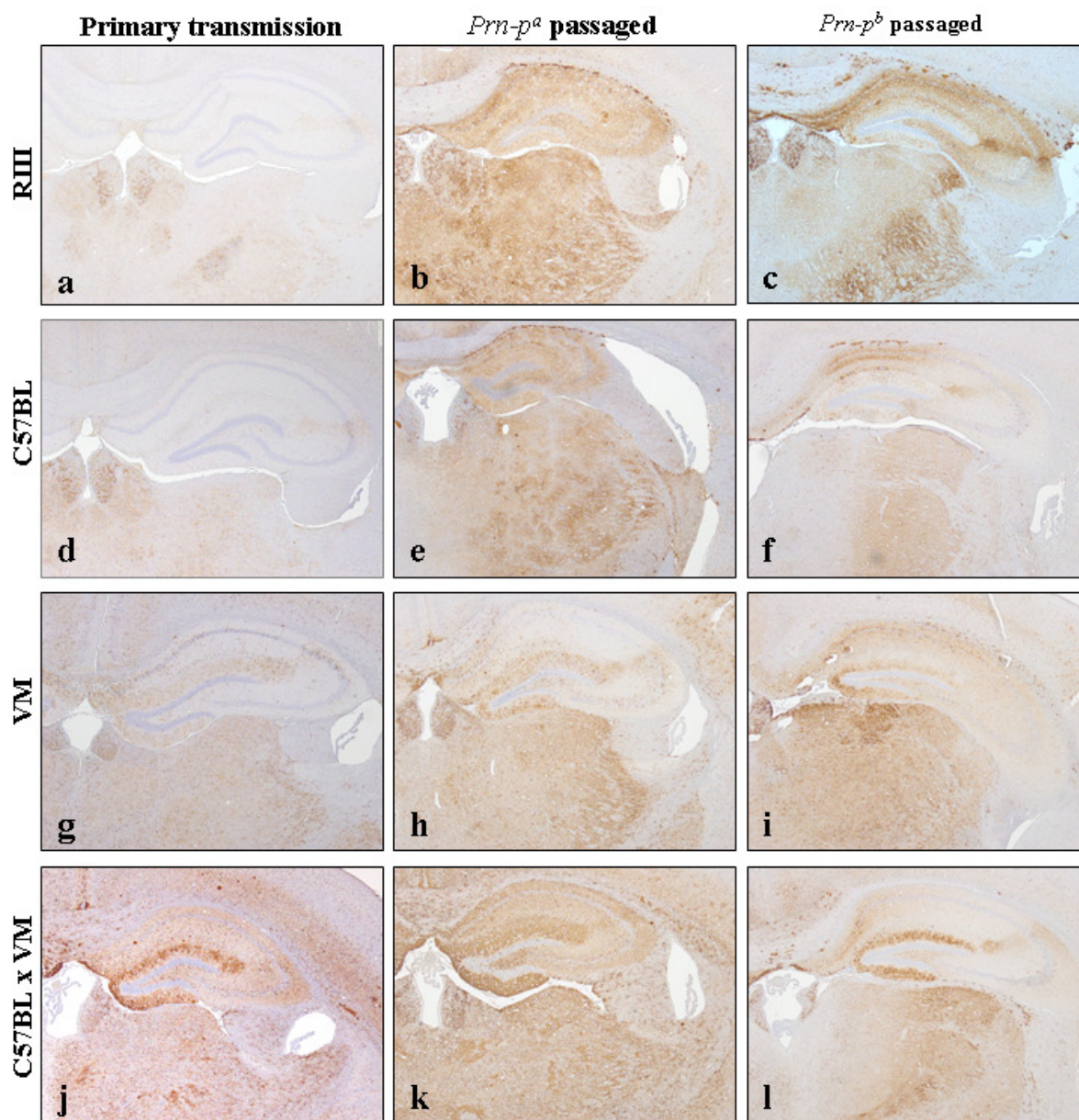
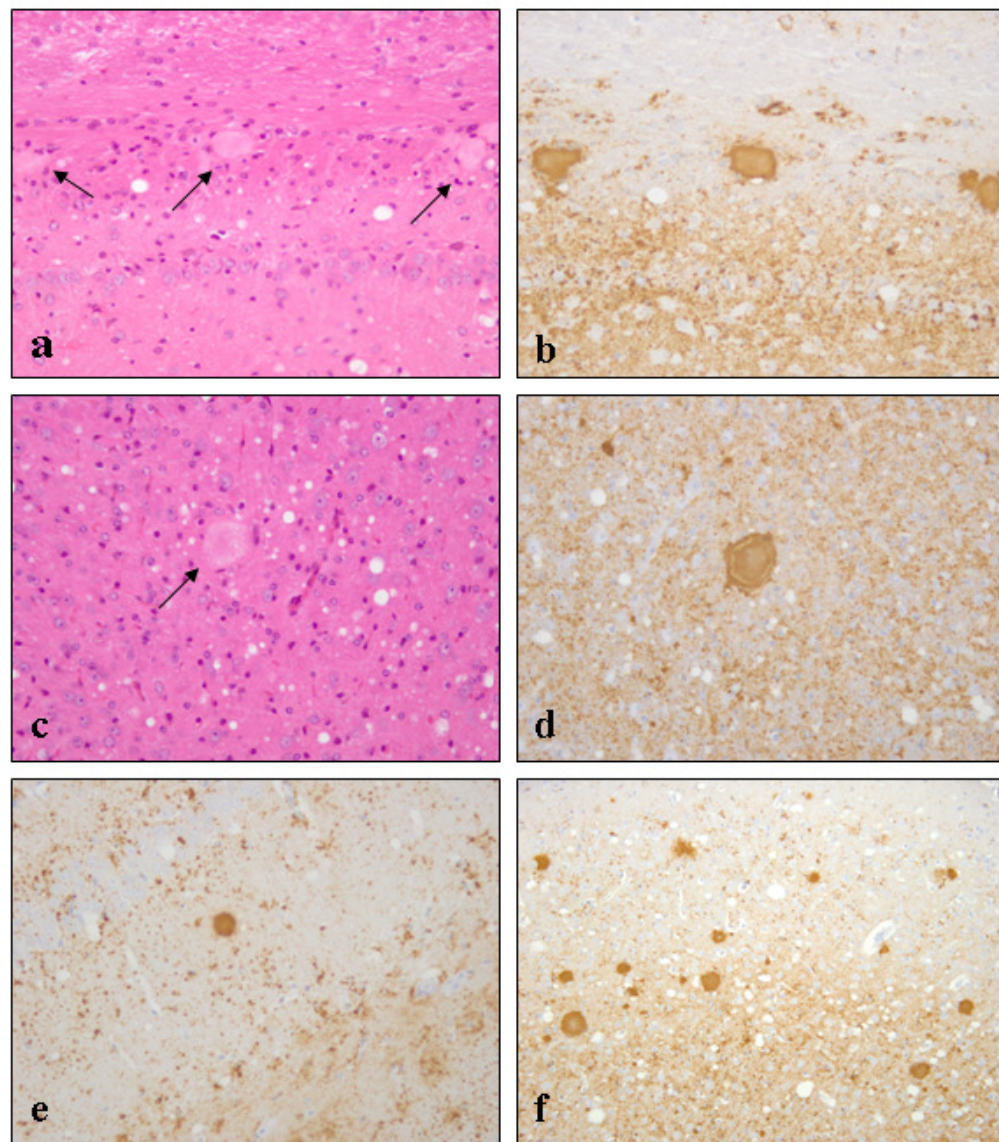


Figure 5.11: Amyloid plaques in mice after serial passage of vCJD brain isolates

Amyloid plaques in all four mouse lines after the passage of vCJD brain isolates in *Prn-p^a* and *Prn-p^b* mice. (a) Haematoxylin and eosin staining showing amyloid plaques (arrows) within the corpus callosum of an RIII mouse and a (b) serial section immunolabelled for the prion protein. (c) Haematoxylin and eosin staining in the thalamus of a C57BL mouse showing the presence of a single amyloid plaque (arrow) and a (d) serial section immunolabelled for the prion protein. (e) PrP deposition in the hippocampus of a VM mouse showing the presence of an amyloid plaque. (f) Amyloid plaques throughout the cerebral cortex of a C57BL x VM mouse. Sections showing PrP immunohistochemistry were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x100 (e, f), x200 (a, b, c, d)



5.4.4 Biochemical isotyping of PK resistant PrP^{Sc}

Results have confirmed that the biochemical phenotype of vCJD is maintained on primary transmission to wild-type mice. To establish whether PrP^{res} type is further conserved on subsequent mouse-to-mouse passage, Western blot analysis was carried out as previously described (Chapter 2, section 2.7) on a minimum of two frozen CNS samples of all mouse lines after two serial passages of a single vCJD brain isolate (CJD 28) in *Prn-p^a* and *Prn-p^b* mice. Western blot analysis was carried out on 5µl extracts of a 10% brain tissue weight to buffer volume (w/v) unless otherwise stated. Mouse samples were run alongside a sCJD MM1 diagnostic brain reference standard and a vCJD diagnostic brain reference standard.

PrP^{res} was readily detected in all mouse CNS extracts analysed. The intensity of the PrP^{res} signal with 5µl mouse CNS extracts was observably higher than those seen in the human CJD reference standard (Figure 5.12) and that of the primary passages (Figure 5.6). If PrP^{res} correlates with infectivity, as has been proposed, this may be an indication of higher levels of infectivity associated with mouse CNS tissue following serial passage of the vCJD brain isolates. All mouse samples were re-analysed with 1µl PrP^{res} extracts. The resulting PrP^{res} banding pattern showed predominance in the diglycosylated form of the protein, typical of vCJD in human CNS tissue (Figure 5.13 and Figure 5.14) and in the mice after primary mouse passage (Figure 5.6). In addition, the appearance of the unglycosylated form as a doublet, composed of distinct upper and lower bands, was also maintained on mouse-to-mouse passage. No significant differences were observed in the PrP^{res} banding pattern between *Prn-p^a* and *Prn-p^b*-passaged isolates or between the first and second passage; however, there was notable variation in the relative abundance of the upper and lower unglycosylated bands (Figure 5.13 and Figure 5.14). Unlike the primary mouse passages, where the lower band of the doublet was found to predominate in *Prn-p^a* mice and the upper band predominated in *Prn-p^b* mice, there appeared to be no simple correlation between the composition of the doublet and the *Prn-p* genotype of the mouse on secondary passage. Regardless of the variation in the unglycosylated doublet, PrP^{res} typing in the mice has provided strong evidence that the biochemical

phenotype of vCJD is conserved in mice after serial mouse passage of vCJD brain isolates.

Figure 5.12: Western blot analysis of PrP^{res} in vCJD brain isolates

Western blot analysis of PrP^{res} in 5µl CNS extracts of an RIII and VM mouse after the 1st mouse-to-mouse passage of brain isolates in C57BL (*Prn-p^a*) mice. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).

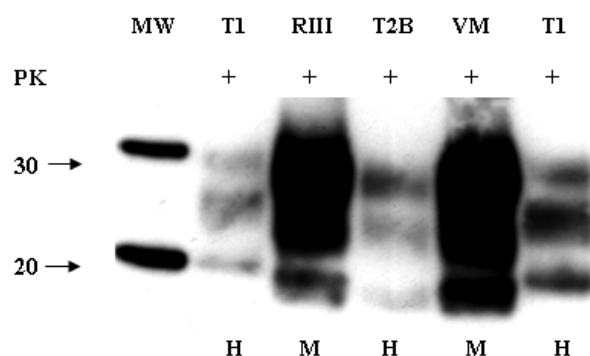


Figure 5.13: Western blot analysis of PrP^{res} in vCJD brain isolates passaged in *Prn-p^a* mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after the 1st and 2nd mouse-to-mouse passage of brain isolates in C57BL (*Prn-p^a*) mice. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).

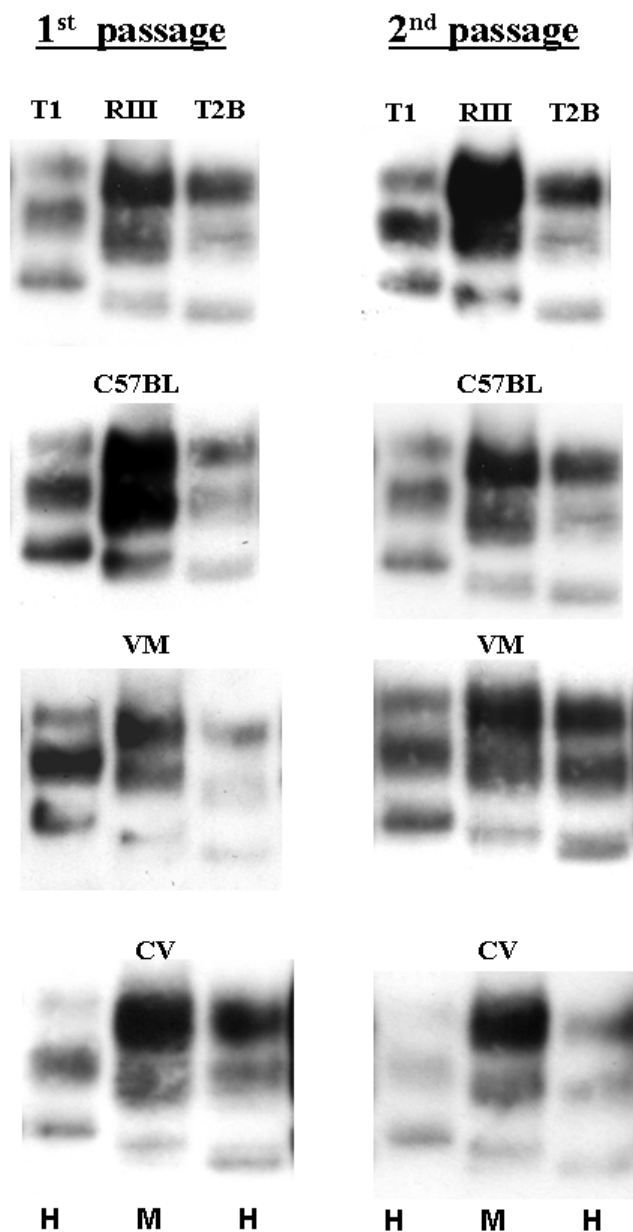
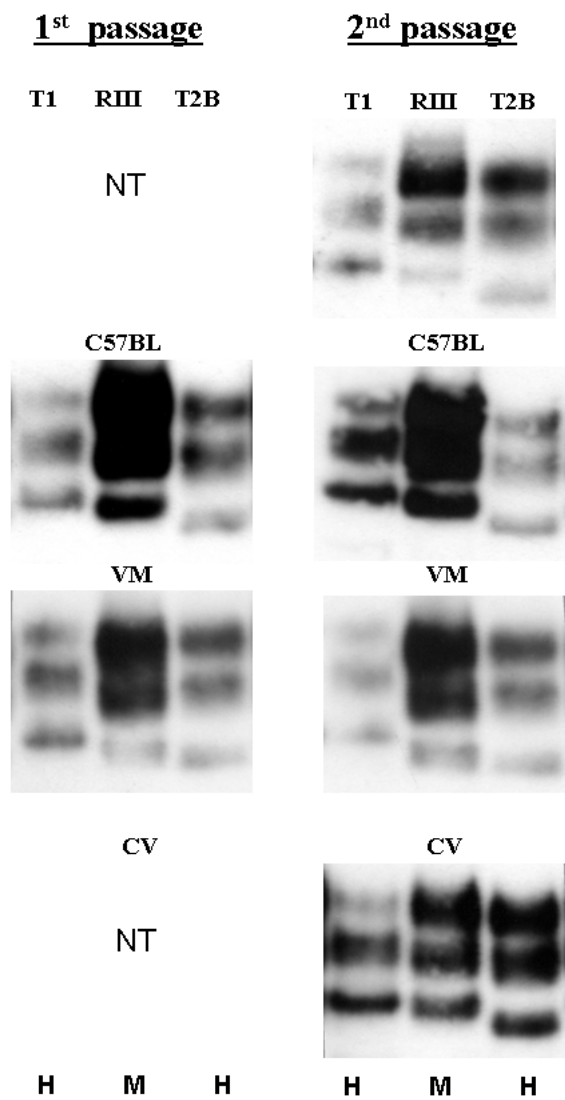


Figure 5.14: Western blot analysis of PrP^{res} in vCJD brain isolates passaged in *Prn-p^b* mice

Western blot analysis of PrP^{res} in CNS samples of RIII, C57BL, VM and C57BL x VM (CV) mice after the 1st and 2nd mouse-to-mouse passage of brain isolates in VM (*Prn-p^b*) mice. Tissues are analysed after digestion with proteinase K. Mouse samples (M) are run alongside a human (H) sCJD MM1 type 1 (T1) and a vCJD diagnostic standard (T2B).



NT- no frozen tissue available for Western blot analysis

5.5 Results Summary

Variant CJD is characterised by a number of unique and highly reproducible biological and biochemical features after transmission into panels of wild-type mice. The consistency in the transmission properties shown by each vCJD brain homogenate supports previous reports that vCJD results from infection with a single major strain of agent. Variant CJD in wild-type mice is characterised by the following features;

- Variant CJD-infected mice develop a progressive clinical disease.
- Clinical signs occur in a consistent order of appearance in the wild-type panel, with the shortest incubation periods in the RIII mouse line with the longest in the C57BL x VM cross.
- Incubation periods differences of over 100 days are observed in mouse lines of the same *Prn-p* genotype.
- The lesion profile in vCJD-infected mice differs between mouse lines of different *Prn-p* genotypes, but within each mouse line there are few differences between isolates derived from different vCJD patients.
- Amyloid plaques are observed in VM mice and C57BL x VM cross after primary transmission and in all mouse lines on subsequent serial mouse passage.
- Molecular PrP^{res} typing in the infected mouse shows conservation in the vCJD type on primary transmission and on further serial passage in mice, which is dominated by the diglycosylated form of the protein.
- Serial mouse passage of vCJD results in the isolation of two distinct mouse-passaged strains, recognised by incubation period in VM mice; a VM-passaged strain and an RIII/C57BL-passaged strain.

5.6 Discussion of experimental results

The aim of this chapter was to complete and further extend the work described in the original vCJD strain typing paper by Bruce and co-workers (Bruce *et al.*, 1997). In the original study, characterisation of the vCJD agent strain was based solely on incubation period data and lesion profiling in RIII mice after the experimental transmission of vCJD from brain homogenate from three vCJD patients (vCJD cases analysed in Bruce *et al* (1997) correspond to patients CJD 28, CJD 29 and CJD 30 analysed in this thesis). These early observations were instrumental in providing evidence that the transmission characteristics of vCJD were indistinguishable from those of BSE, FSE and TSEs in exotic ruminants but differed from the transmission characteristics of sCJD (Bruce *et al.*, 1997). The series of transmissions described in this chapter has confirmed the original observations and extended them in a number of important aspects. Firstly, this study increases the number of transmitted cases in the wild-type mouse panel to 10 and included cases with disease onsets two years after those analysed in the study by Bruce *et al* (1997). Secondly, characterisation of the vCJD agent was extended to include the immunohistochemical and biochemical analysis of the abnormal PrP in the vCJD inoculated mice. Finally, further characterisation of the vCJD agent in the wild-type mouse panel was carried out after two subsequent mouse-to-mouse passages.

5.6.1 Variant CJD cases are associated with a single strain of agent

Primary transmission from each vCJD brain homogenate showed strikingly similar transmission properties for all strain typing parameters considered, consistent with previous studies (Bruce *et al.*, 1997) and indicating that all 10 cases had resulted from infection with the same major strain of agent. In the preliminary studies described by Bruce *et al* (1997), vCJD cases used in the transmissions to wild-type mice were limited to those early on in the epidemic (onsets between 1994 and 1995). In this thesis, analysis of transmissions from a larger number of UK vCJD cases and from cases occurring later in the epidemic (onsets between 1994 and 1997), has shown that the transmission properties of vCJD in mice remain constant, showing no evidence for alterations in the agent strain as the epidemic progressed. It will be interesting to establish whether more recent cases of vCJD or those cases which have

since occurred in other countries share these exact features. These studies are underway as part of the continuing collaboration between the NCJDRSU and the NPU/NPD in the investigations of human TSEs.

As part of this on-going collaboration, a recent study in human transgenic mice, in which the vCJD agent was characterised in patients who were infected as a result of secondary human transmission by blood transfusion, suggests that there is no significant alteration in agent strain following secondary infection via this route (Bishop et al., 2008). Although this study using humanised transgenic mice provides valuable evidence on the vCJD agent acquired through human-to-human transmission, it remains true that agent strain in TSEs is best defined by passage in non-transgenic (wild-type) mice panels. The full characterisation of the vCJD agent described in this chapter will provide a valuable baseline to which data from further vCJD transmissions can be compared.

5.6.2 Regional difference in levels of infectivity in vCJD

Incubation periods in mice are determined by agent strain in combination with host genetic factors, but titre of infectivity can also play an important role. This study found consistently shorter incubation periods in mice following inoculation with vCJD cerebellar tissue when compared to those obtained with inoculations from frontal cortex tissue (Figure 5.1). Although tissues were sampled from different vCJD cases, this outcome suggests that levels of infectivity are consistently higher in the cerebellar cortex than in the frontal cortex. Furthermore, in contrast to homogenate prepared from the frontal cortex tissue, levels of infectivity in the cerebellar homogenate were sufficiently high as to produce incubation period data in all C57BL x VM groups challenged. This may be a further indication that there are regional differences in levels of infectivity in the brain. Although the incubation period data indicates differences in the levels of infectivity between frontal cortex and cerebellum this was not reflected in gross differences in the levels of PrP^{res}, as determined by Western blotting (chapter 3). Furthermore, pathological changes in these different brain regions in vCJD patients also appear to be quantitatively similar; however, deposition of PrP in the cerebellum is often more pronounced compared to

the frontal cortex. In addition, amyloid plaques, a characteristic pathological feature of vCJD, are more frequently observed in the cerebellum compared to the cortical regions of the brain in vCJD (Ironside et al., 2000), perhaps corresponding to the apparent relatively higher levels of infectivity in the cerebellum.

5.6.3 Isolation of distinct mouse-passaged vCJD strains

Incubation period differences of over 100 days were observed between the RIII and C57BL strains of mice, both of which are from the same *Prn-p* genotype (Figure 5.1). This incubation period difference was seen in all seven vCJD transmissions in which both mouse lines were included. This difference in incubation period of vCJD-infected mice of the same *Prn-p* genotype is unusual and has not been observed in transmissions of different strains of scrapie, but is a characteristic feature of BSE in the same mice (Bruce et al., 2002; Ritchie et al., 2009). The possibility that this difference in incubation period may be due to the presence of another minor strain of agent can be excluded, as no differences were found in the agent strain after the serial passage of vCJD brain isolates in RIII and C57BL mice. It is more likely that the differences are due to the direct control on incubation period from a gene or genes other than *prn-p*.

However, further characterisation of the vCJD agent by sub-passage in *Prn-p^a* and *Prn-p^b* mice did result in the isolation of two apparently distinct mouse-passaged strains. Although the evidence indicates that each of the human vCJD brain samples tested contained the same major strain of agent, this does not rule out the presence of other minor strains in these samples. Previous studies of mouse-passaged scrapie have shown that, like viruses, these agents can generate variant “mutant” strains (Bruce and Dickinson, 1987), and it is likely that isolates always contain mixtures of variants. In the case of sheep scrapie and some other TSEs, initial transmission to mice is very inefficient and the biological properties of the isolates only gradually stabilize over several serial mouse passages (Moir Bruce, personal communication). This might suggest that the scrapie strains isolated in mice are derived from minor variant strains present in the sheep, and that there may be further stepwise generation of variants in mice in the process of adaptation. In contrast, vCJD transmits

efficiently to wild-type mice and analysis of incubation period data (Figure 5.8) indicates that the properties stabilize almost immediately on mouse-to-mouse passage, suggesting that the mouse-passaged strains isolated are identical or closely related to strains that were actually present in the human source brains. The ranking of incubation periods in the mouse panel in the primary transmission of vCJD is conserved in the *Prn-p^a* mouse-passaged isolates, but not in the *Prn-p^b* mouse-passaged isolates, further suggesting that the *Prn-p^a* mouse-passaged strain is more representative of the major strain present in humans.

Interestingly, the biological properties of these two mouse-passaged vCJD-related strains were not reflected in differences in the PrP^{res} type found in the brains of the mice replicating these two strains of agent (Figure 5.13 and Figure 5.14). No obvious differences were found in the glycosylation pattern, with the diglycosylated form predominating in all vCJD-passaged mice examined. However, certain differences were observed in the unglycosylated form which, like the primary transmissions, was seen as a doublet. In the primary transmission of vCJD, the upper and lower bands of this doublet appeared to be associated with the different *Prn-p* genotypes in that the lower band is predominant in *Prn-p^a* mice whereas the upper band predominates in *Prn-p^b* mice. These apparent differences in the biochemical profile of PrP^{res} could perhaps be attributed to differences in the polymorphic residues between *Prn-p^a* and *Prn-p^b* that result in different conformation or aggregation states of the misfolded form of the protein. However, this simple relationship did not hold on serial passage of vCJD brain isolates in the mice and so the biochemical basis of this phenomenon and its biological significance remain unclear.

Comparison of the strain typing parameters in the mice showed clear differences between the transmission properties of vCJD and sCJD inoculated mice (chapter 4). The most striking differences were in the development of clinical TSE disease. Almost 100% of mice inoculated with vCJD developed a progressive neurological disease-associated with a TSE, whereas clinical disease in the sCJD inoculated mice was rare. It is interesting that the only evidence of clinical disease in the sCJD inoculated mice resulted from experimental challenge of brain homogenate from the

sCJD MM2 subgroup. This sCJD subgroup shares the most similarities with vCJD. Both diseases occur in the same *PRNP* codon 129 genotype and both have a similar protein conformation, as inferred from the electrophoretic mobility of the unglycosylated fragment (approximately 19kDa) after PK digestion. This may be an indication that strain-specific properties in TSEs may be associated with differences in the glycosylation states of the protein rather than differences in conformation. The relationship between vCJD and sCJD will be addressed in greater detail in the discussion (Chapter 7, section 7.2.4).

In addition to confirming that the agent strain in vCJD differs from that of sCJD, this series of transmissions has confirmed that the transmission properties of vCJD in this mouse panel are indistinguishable from those previously described for BSE, confirming that a BSE-related strain of agent is involved in vCJD. The close relationship between the BSE agent and that of vCJD is addressed in detail in the discussion (Chapter 7, section 7.2.3).

5.7 Chapter conclusion

In conclusion, results from this extensive series of vCJD transmissions to wild-type mice have provided further evidence that vCJD is the result of infection with a single major strain of agent. The data described in this chapter will provide a valuable standard on which to compare future vCJD transmissions experiments, including any future cases of vCJD in the other *PRNP* codon 129 genotypes, in order to determine any changes in agent strain in these different genotypes.

Chapter 6: Detection of infectivity in peripheral neural and lymphoid tissues and biological fluids of sporadic and variant CJD patients by transmission to wild-type mice

6.1 Objective

1. To determine the presence of infectivity within a range of lymphoid and neural tissues from sCJD and vCJD patients.
2. To determine the presence of infectivity within bone marrow, buffy coat, plasma and cerebrospinal fluid from vCJD patients. In addition, the presence of infectivity in buffy coat and plasma from sCJD patients will be investigated.
3. To characterise the agent strain in lymphoid and neural tissues of CJD patients and to determine whether agent strain characteristics are subject to any tissue specific alterations.

6.2 Introduction

TSEs are characterised by vacuolar degeneration in CNS tissues and the accumulation in the brain of the abnormal and disease-associated form of the prion protein. Results from previous chapters have confirmed that TSE infectivity in brain tissue of vCJD (chapter 5) and to a lesser extent sCJD (chapter 4) patients can be transmitted successfully to wild-type mice. However, tissue involvement, as judged by the deposition of the abnormal form of the prion protein in TSEs is not confined to the CNS. Abnormal accumulations of the prion protein have been described in peripheral tissues in scrapie in sheep (van Keulen et al., 1996; van Keulen et al., 1999), chronic wasting disease in deer (Sigurdson et al., 1999; Sigurdson et al., 2001), as well as a number of animal models of TSEs (Kimberlin and Walker, 1979; Maignien et al., 1999; Beekes and McBride, 2000). In human TSEs, the deposition of PrP^{Sc} in peripheral lymphoid tissues is a characteristic and diagnostic feature of vCJD and has indicated a widespread involvement of lymphoid tissues in vCJD at the end-stage of disease (Head et al., 2004b). Furthermore, infectivity in vCJD spleen and tonsil tissue has been confirmed by bioassays in RIII mice, which have indicated

infectivity levels in these tissues a hundred to a thousand times lower than those demonstrated in brain (Bruce et al., 2001). In addition to lymphoreticular tissues, immunohistochemistry for PrP has demonstrated abnormal PrP positivity in a variety of peripheral nervous tissues including trigeminal, dorsal root and autonomic ganglia (Haik et al., 2004; Head et al., 2004b). Further investigations using highly sensitive immunohistochemical and Western blotting techniques have also identified PrP^{Sc} within the pituitary gland (Peden et al., 2007), peripheral motor nerve fibres in skeletal muscle (Glatzel et al., 2003; Peden et al., 2006) and more recently for the first time in dura mater, liver, pancreas, kidney, ovary, uterus and skin in vCJD (Notari et al., 2010). Thus, the number of tissues and organs showing the presence of the abnormal prion protein may be greater than previously thought. In contrast, the distribution of PrP^{Sc} pathology is thought to be largely restricted to the CNS system in cases of sCJD (Head et al., 2004b). However, findings from studies using a highly sensitive Western blot technique have shown detectable levels of PrP^{res} in the pituitary gland (Peden et al., 2007), spleen and intramuscular nerve fibres (Glatzel et al., 2003; Peden et al., 2006) in a proportion of sCJD patients suggesting that there may also be a more limited peripheral involvement in some cases of sCJD.

Reports of PrP^{Sc} in tissues other than brain are generally described in vCJD patients who had died following the development of clinical symptoms of disease. However, in a retrospective review of tissues removed from vCJD patients prior to the onset of clinical disease, PrP positivity was detected in appendix samples surgically removed from two patients that went on to develop clinical vCJD eight months and two years after their appendectomies (Hilton et al., 1998). The detection of PrP^{Sc} in lymphoid tissues prior to the onset of clinical disease has been widely reported in both natural and experimental models of scrapie and confirms earlier findings from bioassay in mice which demonstrated that replication of the TSE agent occurs in the lymphoreticular system before detection in the CNS (Dickinson and Fraser, 1969; Kimberlin and Walker, 1979; Kimberlin and Walker, 1989a). The demonstration of PrP^{Sc} in the appendix taken from individuals who went on to develop vCJD suggests that there may be a similar pathogenesis in vCJD. More recently, the apparent transmission of vCJD infectivity to four individuals by blood transfusion, associated

with transfusions of blood collected from vCJD patients during the asymptomatic phase of the disease (Llewelyn et al., 2004; Peden et al., 2004; Wroe et al., 2006; Health Protection Agency., 2009) and the detection of infectivity in a haemophiliac patient after treatment with UK-sourced plasma products prepared from “vCJD-implicated batches” (Peden et al., 2010), further supports the proposal that infectivity is present in peripheral tissues and biological fluids much earlier than in the brain.

The accumulation of the abnormal prion protein outside the brain has raised concerns about iatrogenic transmission risks associated with contamination of surgical instruments, tissue transplantation and treatment with pharmaceuticals prepared from human tissue. Furthermore, the cases of vCJD infection, associated with contaminated blood products collected from vCJD patients during the asymptomatic phase of the disease (Llewelyn et al., 2004; Peden et al., 2004; Wroe et al., 2006; Health Protection Agency., 2009; Peden et al., 2010), highlights the risks and concerns over the secondary human transmission of vCJD. An additional concern is the theoretical possibility of alterations in agent strain on secondary transmission from human-to-human which may result in a modified disease phenotype and biochemical profile which may have implications for the diagnosis of such cases.

To date, PrP^{Sc} has been reported in a wide range of peripheral tissues and organs in vCJD; however, it remains that the published demonstration of infectivity in tissues other than brain has been limited spleen and tonsil. This chapter aims to extend the evidence for infectivity in other vCJD lymphoid (lymph node and appendix) and neural tissues (spinal cord, DRG, TRG and peripheral nerve) in which the presence of the pathological prion protein has been confirmed by immunohistochemistry and Western blot assays. In addition, this study extends the investigation to include blood fractions (buffy coat and plasma), CSF and bone marrow, in which the presence of PrP^{Sc} has not been demonstrated by such conventional methods but in which the demonstration of infectivity could play an important role in the risk assessment for certain medical procedures such as blood transfusion and bone marrow transplantation. Furthermore, this chapter aims to investigate whether a similar

involvement of the lymphoid system occurs at the end stage of disease in sCJD, by extending the analysis to include transmissions from sCJD spleen. Finally, this chapter also provides the complete incubation period data, lesion profiles and immunohistochemical analysis of the abnormal prion protein accumulation in mice following transmission from these tissue homogenates to establish whether the agent strain is subject to any tissue specific alterations.

6.3 Experimental results

Forty-one transmission experiments were carried out with tissue inocula prepared from specified peripheral tissues, neural tissues and biological fluids from four sCJD cases (CJD 10, CJD 11, CJD 15 and CJD 18) and seven vCJD cases (CJD 31, CJD 35, CJD 36, CJD 37, CJD 38, CJD 39 and CJD 40), as summarised in Table 6.1. Tissue homogenates were prepared and inoculated intracerebrally into RIII (*Prn-p^a*) and VM (*Prn-p^b*) mice as described in Chapter 2, section 2.3. Frozen CNS samples were collected from a small number of mice inoculated with vCJD spleen and tonsil homogenate.

Transmission of a TSE, as judged by the development of neurological signs associated with TSE and the presence of vacuolar pathology was seen in some mice inoculated with vCJD spleen, tonsil, lymph node, peripheral nerve, DRG, TRG and spinal cord samples, but with varying degrees of efficiency between the different tissue homogenates. Subsequent immunohistochemical detection for PrP also showed evidence of positive transmission in mice challenged with vCJD appendix. In contrast, mice inoculated with vCJD bone marrow, buffy coat, plasma and CSF showed no evidence of a TSE. All sCJD transmissions were negative with the exception of one mouse which showed TSE type pathology after challenged with a sCJD buffy coat sample. Results from the experimental challenge of the different tissue types are detailed below.

Table 6.1: Tissues/fluids experimentally inoculated into wild-type mice

Case	Diagnosis	Tissues used as inoculum	Mouse lines challenged
CJD 10	sCJD MM1	BC, SPL	RIII, VM
CJD 11	sCJD MM1+2A	BC, PL	RIII, VM
CJD 15	sCJD MV1	BC	RIII, VM
CJD 18	sCJD MV2A	BC, PL	RIII, VM
CJD 31	vCJD	BC, PL, SPL, TONS	RIII, VM
CJD 35	vCJD	BC, PL, TONS	RIII, VM
CJD 36	vCJD	BC, PL	RIII, VM
CJD 37	vCJD	BC, PL	RIII, VM
CJD 38	vCJD	APP, BM, CSF, DRG, LN, PN, SC, TRG	RIII
CJD 39	vCJD	APP, BM, DRG, LN, PN, SC, TRG	RIII
CJD 40	vCJD	APP, BM, CSF, DRG, LN, PN, SC, TRG	RIII

APP – appendix, BC – buffy coat, BM – bone marrow, CSF – cerebrospinal fluid, DRG – dorsal root ganglia, LN – lymph node, PL – plasma, PN – peripheral nerve, SC – spinal cord, SPL – spleen, TONS – tonsil, TRG – trigeminal ganglia

6.3.1 Spleen tissue

Two transmission experiments were carried out with inoculum prepared from spleen tissue from one sCJD (CJD 10) case and one vCJD case (CJD 31). The incidence of clinical signs of disease and TSE-specific vacuolar pathology in the inoculated mice, and results from the analysis of PrP accumulation in some mice from each experimental group are shown in Table 6.2.

Clinical TSE disease was observed in a proportion of the RIII and VM mice following experimental challenge with vCJD spleen homogenate, resulting in incubation periods of 490 ± 18 and 659 ± 11 respectively (Table 6.2). These incubation periods were longer than those observed for vCJD brain homogenate in the same mouse lines, as detailed in chapter 5. In contrast, no evidence of a neurological disease indicative of a TSE was observed in any RIII or VM mouse challenged with the sCJD spleen homogenate. Further histological examination also failed to show any evidence of TSE-specific vacuolar pathology in the sCJD inoculated mice (Table 6.2).

In general, vacuolar degeneration was observed in RIII and VM mice that had developed clinical TSE disease, confirming the successful transmission of TSE infectivity from vCJD spleen. Only a single VM mouse produced vacuolar pathology in the absence of any clinical signs of disease (Table 6.2). Patterns of vacuolar pathology in the clinically affected mice differed between the two mouse PrP genotypes (Figure 6.1). The lesion profile in the RIII mouse line was characterised by the targeting of a mild-moderate vacuolar pathology in the medulla oblongata (G1) and the hypothalamus (G4) whereas, the pattern of vacuolar degeneration in the VM mouse line resulted in a more widespread distribution of mild-moderate vacuolar pathology throughout the grey matter scoring regions of the brain. The patterns of vacuolar pathology in the clinically affected RIII and VM mice were closely similar to those seen in the same mouse lines inoculated with vCJD brain homogenate as described in chapter 5.

Table 6.2: Primary transmission from vCJD and sCJD spleen homogenates

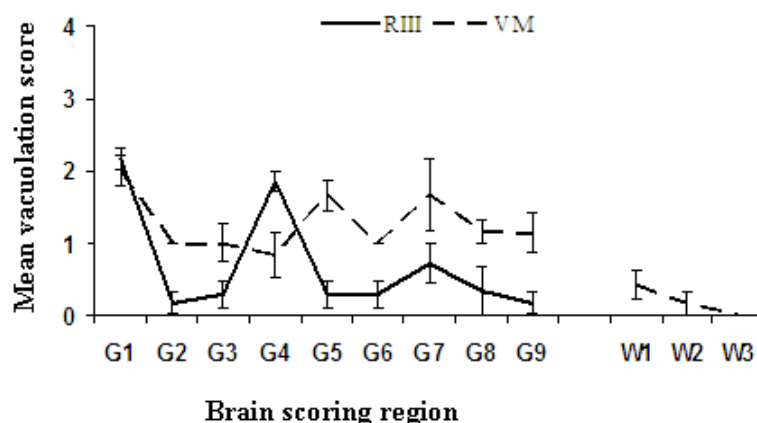
Clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in mice intracerebrally inoculated with spleen homogenate from a sporadic CJD and variant CJD patient. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Mean incubation periods in days are shown in brackets \pm S.E.M.

	Mouse line					
	RIII			VM		
Source	Clin	Vac path	PrP path	Clin	Vac path	PrP path
CJD 10 (sCJD)	0/8	0/8	0/5	0/5	0/5	0/5
CJD 31 (vCJD)	8/19 (490 \pm 18)	8/19	*7/7	7/12 (659 \pm 11)	8/12	*7/7

* mice analysed had a positive score for vacuolar pathology sacrificed with clinical disease

Figure 6.1: Lesion profiles for mice inoculated with vCJD spleen homogenate

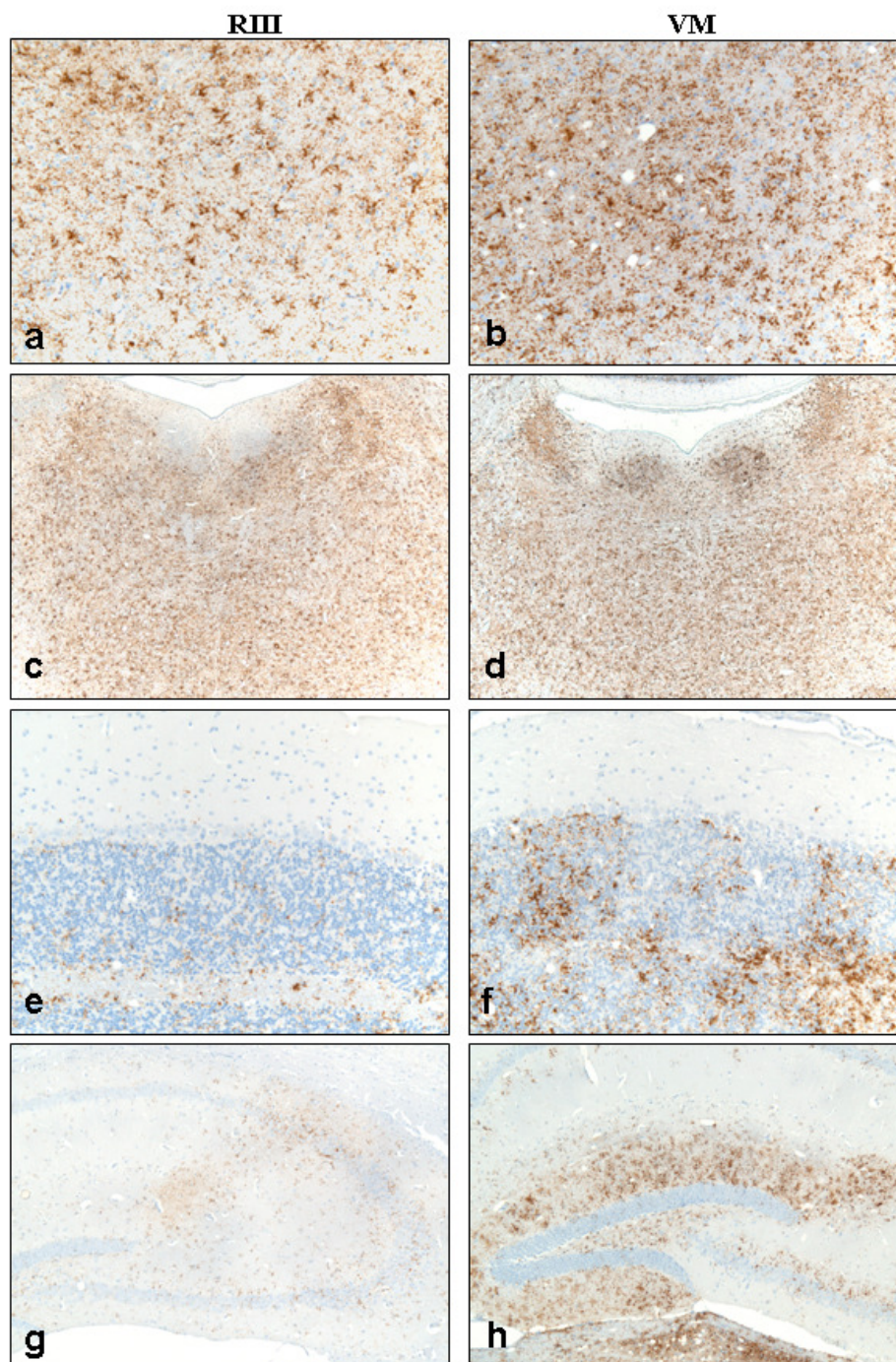
Mean lesion profiles are constructed from a minimum of five RIII mice (solid line) and five VM mice (dashed line) scoring positive for both clinical TSE disease and TSE-specific vacuolar pathology. (G) grey matter scoring regions, (W) white matter scoring regions of the mouse brain. Mean lesion profiles are shown with standard error bars (\pm S.E.M).



Immunohistochemistry for PrP was carried out on 5µm formalin fixed, formic acid treated brain sections from seven RIII and seven VM mice that had received a positive score for both clinical and vacuolar signs of TSE following challenge with the vCJD spleen homogenate. Immunolabelling was carried out using the CSA detection method in combination with the monoclonal anti-PrP antibody 6H4 as described in Chapter 2, section 2.6.4. All mice analysed showed an intense and widespread accumulation of PrP in the mouse brain, further confirming the successful transmission of a TSE from the vCJD spleen homogenate. Pattern of PrP accumulation in the mouse brain was similar in both mouse lines, with a peri-cellular pattern of PrP staining, most prominent throughout the thalamus (Figure 6.2a and b), medulla (Figure 6.2c and d) and around areas of intense vacuolation. PrP deposition was also observed in the granular layer and to a lesser extent the molecular layer of the cerebellum (Figure 6.2e and f). Subtle differences in staining pattern were observed in the hippocampal region between the RIII and VM inoculated mice, a feature also observed in the experimental transmission of vCJD brain homogenates in the same mouse lines. The RIII mice showed a granular deposition of PrP targeting the CA2 region of the hippocampus, whereas the VM mice showed a more intense deposition of PrP targeting the dentate gyrus (Figure 6.2g and h). In general, levels of PrP immunostaining appeared higher in the RIII (*Prn-p^a*) mice compared to the VM (*Prn-p^b*) mice. Amyloid plaques were not observed in the mice. Immunohistochemistry on five RIII and five VM mice inoculated with sCJD spleen homogenate with survival times over 750dpi showed no detectable PrP accumulation in the brain.

Figure 6.2: PrP deposition in mice inoculated with vCJD spleen homogenate

Heavy granular deposits of PrP targeting the thalamus and medulla in **(a, c)** RIII and **(b, d)** VM mice. **(e)** Small granular deposits of PrP targeting the granular layer of the cerebellum in RIII mice with a more intense labelling pattern observed in the granular layer of **(f)** VM mice. **(g)** Granular deposition of PrP targeting the CA2 region of the hippocampus of RIII mice. **(h)** Heavy peri-cellular labelling of PrP in the dentate gyrus region of the hippocampus in a VM mouse. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x50 (c, d), x100 (g, h), x200 (a, b, e, f).



6.3.2 Tonsil tissue

Two transmission experiments were carried out with homogenate prepared from tonsil tissue sampled from two vCJD cases (CJD 31 and CJD 35). The incidence of clinical signs of disease and TSE-specific vacuolar pathology in the inoculated mice, and results from the analysis of PrP accumulation in some mice from each experimental group are shown in Table 6.3.

Clinical TSE disease was observed in some RIII and VM mice inoculated with both tonsil homogenates (Table 6.3). Mean incubation periods differed between the two homogenates, suggesting that titres of infectivity may be subject to case-to-case or sample-to-sample variation. One tonsil homogenate (CJD 35) produced mean incubation periods that were comparable to those observed in spleen, suggesting similar levels of infectivity present in these different lymphoid inocula.

Only one tonsil homogenate (CJD 35) produced sufficient mice with both clinical and histological evidence of a TSE for the construction of a lesion profile. Vacuolar degeneration in RIII mice showed a consistent pattern to that of vCJD spleen in the same mouse line, characterised by the targeting of a mild-moderate vacuolar pathology in the medulla (G1), hypothalamus (G4) and septum (G7) (Figure 6.3). A different pattern was observed in the VM mouse line with a more widespread distribution of vacuolar pathology throughout the grey matter scoring regions of the brain (Figure 6.3), mirroring the transmission properties of vCJD spleen tissue in the same mouse line.

Table 6.3: Primary transmission from vCJD tonsil homogenates

Clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in mice intracerebrally inoculated with vCJD tonsil homogenates. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Mean incubation periods in days are shown in brackets \pm S.E.M.

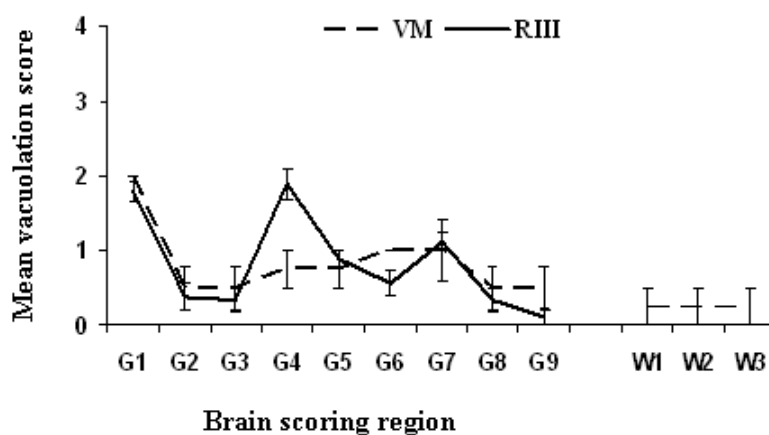
	Mouse line					
	RIII			VM		
Source	Clin	Vac path	PrP path	Clin	Vac path	PrP path
CJD 31	2/4 (490,513)	2/4	2/4	‡4/5 (652 \pm 55)	4/5	4/5
CJD 35	9/19(489 \pm 17)	12/19	*5/5	5/21(524 \pm 22)	9/21	*5/5

* mice analysed had a positive score for vacuolar pathology sacrificed with clinical disease

‡ Incubation period calculated from only four animals in this group which showed a positive score for clinical and vacuolar pathology

Figure 6.3: Lesion profiles for mice inoculated with vCJD tonsil homogenate

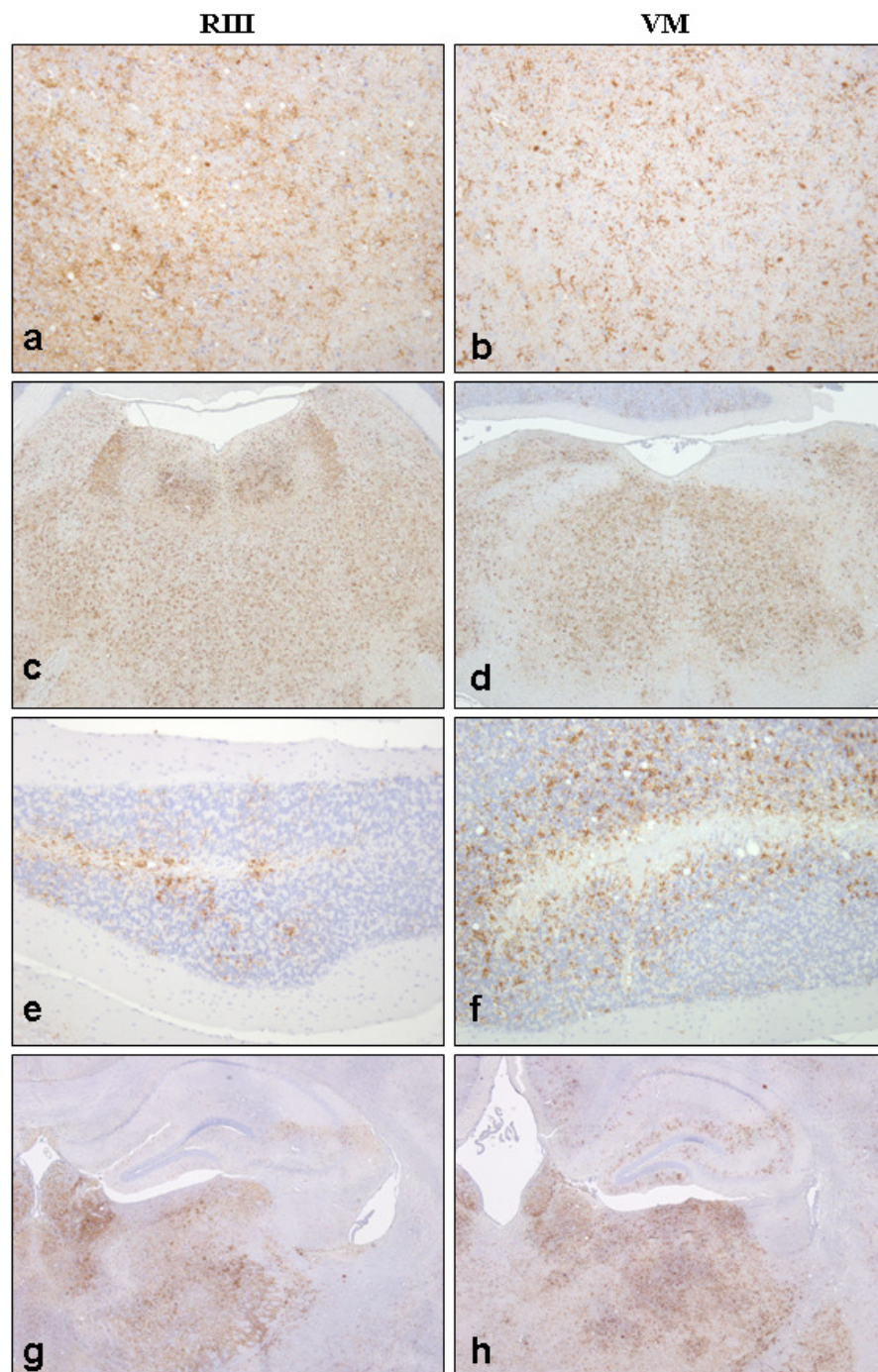
Lesion profiles are constructed from a minimum of five RIII mice (solid line) and five VM mice (dashed line) scoring positive for both clinical TSE disease and TSE-specific vacuolar pathology following inoculation with homogenate from patient CJD 35. (G) grey matter scoring regions, (W) white matter scoring regions of the mouse brain. Mean lesion profiles are shown with standard error bars (\pm S.E.M).



All mice analysed with a positive score for both clinical and vacuolar signs of a TSE showed an intense and widespread deposition of PrP in the brains of the mice, further confirming the transmission of infectivity from vCJD tonsil tissue. Patterns of deposition were similar between the different mouse genotypes and closely resembled that produced by vCJD spleen in the same mouse lines. Intense pericellular deposits of PrP were observed throughout the thalamus (Figure 6.4a and b), hypothalamus and medulla (Figure 6.4c and d) as well as around areas of severe vacuolation. PrP deposition was also observed in the cerebral cortex and in the granular layer and to a lesser extent the molecular layer of the cerebellum (Figure 6.4e and f). The subtle differences in staining pattern within the hippocampal region between the RIII and VM inoculated mice, a feature also observed in the experimental transmission of vCJD spleen and brain isolates in the same mouse lines, were also observed following challenge with vCJD tonsil isolates (Figure 6.4g and h). As described with vCJD spleen, levels of PrP immunostaining appeared higher in the RIII (*Prn-p^a*) mice compared to the VM (*Prn-p^b*) mice. Amyloid plaques, a characteristic feature in the brain of vCJD patients, were not observed in the mice. The two mice analysed with no evidence of clinical disease or vacuolar pathology also showed no evidence of PrP accumulation.

Figure 6.4: PrP deposition in mice inoculated with vCJD tonsil homogenate

Heavy granular deposits of PrP within the thalamus of a (a) RIII and (b) VM mouse. Similar PrP deposition within the medulla in an (c) RIII and (d) VM mouse. Small granular deposits of PrP targeting the granular layer of the cerebellum in an (e) RIII mouse with a more intense labelling pattern observed in the granular layer of a (f) VM mouse. (g) Granular deposition of PrP targeting the CA2 region of the hippocampus of an RIII mouse. (h) Heavy peri-cellular labelling of PrP in the dentate gyrus region of the hippocampus in a VM mouse. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x50 (g, h), x100 (c, d), x200 (a, b, e), x400 (f).



6.3.3 Lymph node tissue

Experimental transmission of homogenates prepared from tissue samples from a cervical lymph node from three vCJD cases (CJD 38, CJD 39 and CJD 40) were carried out in RIII mice. The incidence of clinical signs of disease and TSE-specific vacuolar pathology in inoculated mice, and results from the accumulation of PrP in mice from each experimental group are shown in Table 6.4.

Clinical TSE disease was observed in some mice challenged with two of the vCJD lymph node inocula (CJD 38 and CJD 39), with one producing a mean incubation period of 702 days in the mice, much longer than the incubation periods reported with vCJD spleen and tonsil in the same mouse line. This indicates that infectivity levels in the cervical lymph node are lower than those found in spleen and tonsil. No evidence of clinical TSE disease, vacuolar pathology or PrP accumulation was observed in mice inoculated with the third lymph node homogenate (CJD 40). The variation observed in the efficiency of transmission between the three vCJD lymph node inocula suggests that titres of infectivity differed between these three tissue homogenates.

Vacuolar pathology was observed in only a small proportion of the RIII mice (eight out of 64) surviving over 250dpi; however, the presence of vacuolar pathology in these mice confirms the presence of infectivity in vCJD cervical lymph node. Inocula from CJD 38 produced sufficient mice showing clinical TSE disease and vacuolar degeneration in the brain for the construction of a lesion profile. Pattern of vacuolar pathology in the clinically affected RIII mice showed similarities to that of vCJD spleen and tonsil in the same mouse line, in particular the peak in vacuolar intensity in the hypothalamus (G4) of the mice (Figure 6.5). The similarity in the targeting and severity of vacuolar pathology in RIII mice inoculated with vCJD spleen, tonsil and lymph node isolates indicates close similarities in the transmission properties in these three lymphoid tissues.

Table 6.4: Primary transmission from vCJD cervical lymph node homogenates

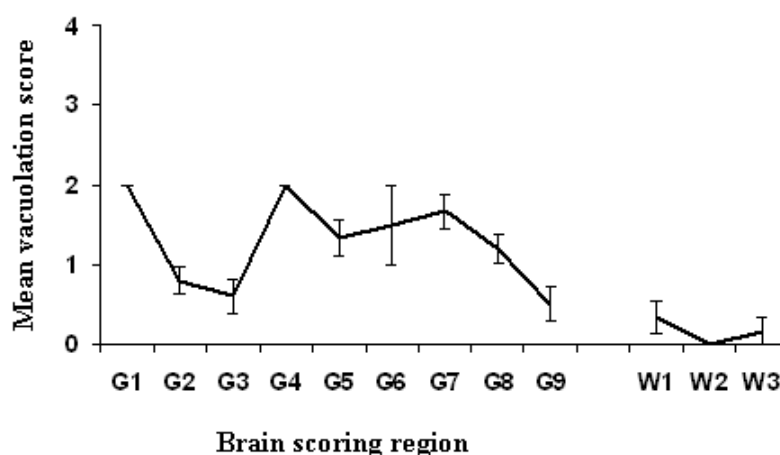
Clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in RIII mice intracerebrally inoculated with vCJD lymph node homogenates. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Mean incubation periods in days are shown in brackets \pm S.E.M.

	Mouse line		
	RIII		
Source	Clin	Vac path	PrP path
CJD 38	6/20 (702 \pm 78)	6/20	*5/5
CJD 39	1/22 (426)	2/22	6/22
CJD 40	0/22	0/22	0/22

* mice analysed had a positive score for vacuolar pathology sacrificed with clinical disease

Figure 6.5: Lesion profiles for mice inoculated with vCJD lymph node homogenate

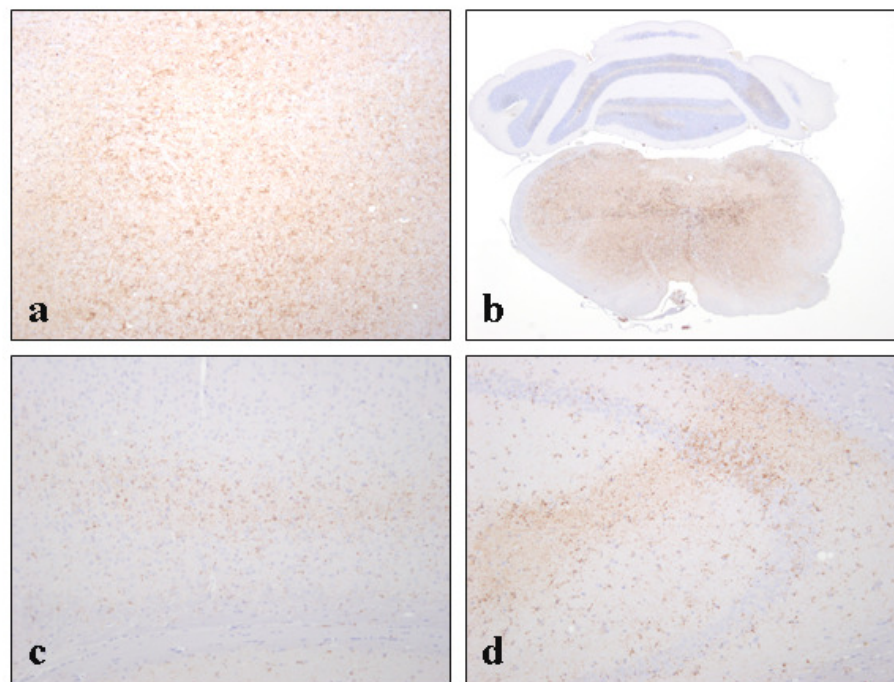
Lesion profiles are constructed from six RIII mice scoring positive for both clinical TSE disease and TSE-specific vacuolar pathology following inoculation with homogenate from CJD 38. (G) grey matter scoring regions, (W) white matter scoring regions of the mouse brain. Mean lesion profiles are shown with standard error bars (\pm S.E.M).



All mice examined by immunohistochemistry which had received both a positive score for clinical and vacuolar signs of a TSE also showed the deposition of PrP in the brain, further confirming the transmission of infectivity from vCJD cervical lymphoid tissue. In addition, PrP deposits were detected in four mice that showed no evidence of vacuolar pathology (Table 6.4, patient CJD 39). The targeting of PrP pathology mirrored that produced by other lymphoid tissues in the same recipient mice with intense peri-cellular deposits of PrP throughout the thalamus, (Figure 6.6a), medulla (Figure 6.6b) and around areas of severe vacuolation. PrP deposition was also observed in the cerebral cortex (Figure 6.6c) and in the granular and molecular layers of the cerebellum. PrP deposition targeting the CA2 region of the hippocampus, a characteristic feature of transmission from the other lymphoid tissues was also observed in the mice (Figure 6.9d).

Figure 6.6: PrP deposition in mice challenged with vCJD lymph node homogenate

Deposition of PrP in RIII mice which showed clinical and histological evidence of a TSE following inoculation with homogenate prepared from a vCJD cervical lymph node. Intense granular and peri-cellular deposits of PrP throughout the **(a)** thalamus and **(b)** medulla. **(c)** Fine granular deposits of PrP in the cerebral cortex. **(d)** PrP deposition targeting the CA2 region of the hippocampus. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x25 (b), x100 (a), x200 (c, d)



6.3.4 Appendix tissue

Inoculation of homogenate prepared from appendix tissue sampled from three vCJD patients (CJD 38, CJD 39 and CJD 40) was carried out in RIII mice. Table 6.5 details the incidence of clinical signs of disease, TSE-specific vacuolar pathology and deposition of PrP in each experimental group.

No clinical signs of a neurological disease related to a TSE were observed in any of the RIII mice following inoculation with appendix homogenate (clinical signs were observed in a single RIII mouse but histological analysis failed to confirm a TSE disease). In addition, no TSE-specific vacuolar pathology was observed in any mouse following challenge with appendix homogenate (Table 6.5).

The lack of any vacuolar pathology in the mice could indicate that there is no transmission of infectivity from vCJD appendix tissue. However, immunohistochemical analysis for the prion protein in all mice inoculated, confirmed the presence of PrP accumulation in 6 out of the 31 mice challenged with homogenate prepared from appendix tissue sampled from patient CJD 38 and CJD 39 (Table 6.5). This provided the first direct evidence for the successful transmission of infectivity from vCJD appendix. One mouse showed PrP deposition with a closely similar distribution and level of intensity to that described for mice infected with other vCJD tissue homogenates (Figure 6.7a-c). The remaining five RIII mice showed a more restricted pattern of PrP pathology, mainly targeting the medulla (Figure 6.7d) and thalamus of the mouse brain. No evidence of PrP accumulation was seen in mice challenged with appendix homogenate from patient CJD 40.

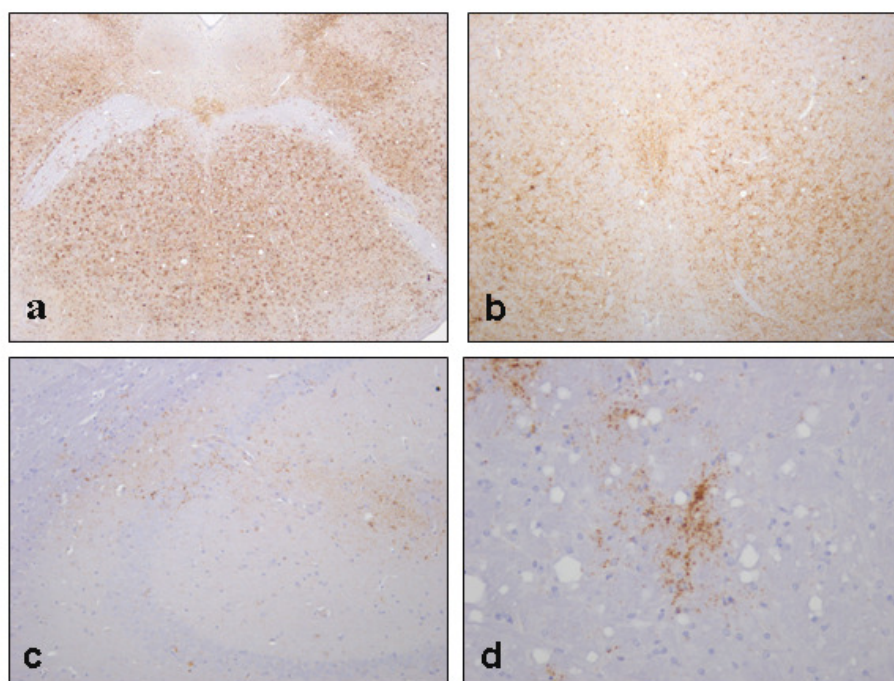
Table 6.5: Primary transmission from vCJD appendix homogenates

Incidence of clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in RIII mice intracerebrally inoculated with vCJD appendix. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Mouse line		
	RIII		
Source	Clin	Vac path	PrP path
CJD 38	0/12	0/12	2/12
CJD 39	1/19	0/19	4/19
CJD 40	0/21	0/21	0/21

Figure 6.7: PrP deposition in RIII mice inoculated with vCJD appendix homogenate

Deposition of PrP in RIII mice following inoculation with homogenate prepared from a vCJD appendix tissue. Intense granular and peri-cellular deposits of PrP throughout the **(a)** medulla and **(b)** thalamus. **(c)** PrP deposition targeting the CA2 region of the hippocampus. **(d)** Small intense deposits of PrP targeting the medulla in the presence of aging vacuolation. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x50 (a, b), x200 (c), x400 (d).



6.3.5 Peripheral nerve tissue

Inoculation of peripheral nerve homogenates prepared from three vCJD patients (CJD 38, CJD 39 and CJD 40) was carried out in RIII mice. Table 6.6 details the incidence of clinical signs of disease, TSE-specific vacuolar pathology and deposition of PrP in each experimental group.

Clinical TSE disease was observed in one RIII mouse following inoculation of three vCJD peripheral nerve homogenates. The presence of TSE-specific vacuolar degeneration in this mouse confirmed the diagnosis of a TSE (Table 6.6). No evidence of vacuolar pathology was observed in any other RIII mouse challenged with the three peripheral nerve inocula. With insufficient mice available for the construction of a lesion profile, it was not possible to compare the single mouse profile from the vCJD peripheral nerve with lesion profiles from other peripheral and neural tissues. The lesion profile is therefore not a relevant parameter in the strain typing of vCJD in peripheral nerve tissue investigated in this study.

Immunohistochemistry on all mice inoculated confirmed the transmission of infectivity from all three vCJD peripheral nerve homogenates, with the demonstration of PrP accumulation in a small number of the mice tested (Table 6.6). The intensity of the PrP labelling varied between the individual mice; however, the general pattern of labelling closely resembled that of vCJD lymphoid homogenates inoculated in RIII mice (Figure 6.8). This suggests that in those mice which do show positive transmission after challenge with vCJD peripheral nerve, the transmission properties are consistent with those found in mice challenged with other vCJD tissue homogenates. These challenges indicate that transmission of infectivity from peripheral nerve can occur but it is rare or inefficient.

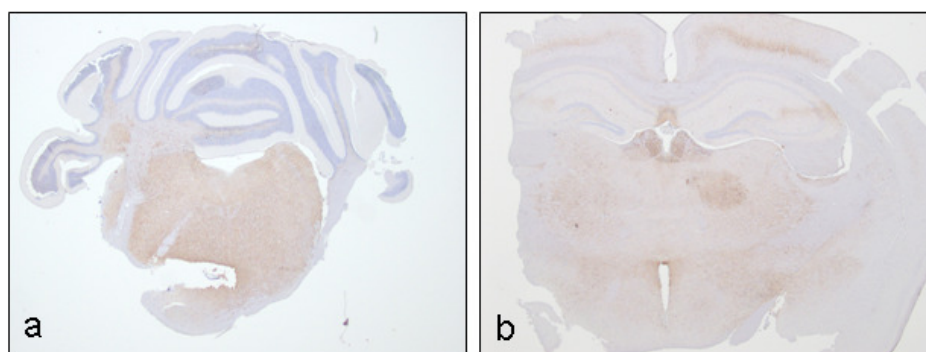
Table 6.6: Primary transmission from vCJD peripheral nerve homogenates

Incidence of clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in RIII mice intracerebrally inoculated with vCJD peripheral nerve homogenates. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Incubation periods in days are shown in brackets.

	Mouse line		
	RIII		
Source	Clin	Vac path	PrP path
CJD 38	1/16 (547)	1/16	4/16
CJD 39	0/22	0/22	1/22
CJD 40	0/23	0/23	1/23

Figure 6.8: PrP deposition in an RIII mouse inoculated with vCJD peripheral nerve homogenate

Intense granular and peri-cellular deposits of PrP throughout the (a) medulla and (b) thalamus, hypothalamus, hippocampus, cerebral cortex of an RIII mouse challenged with a vCJD peripheral nerve homogenate. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification x25



6.3.6 Dorsal root ganglia tissue

Three transmission experiments were carried out in RIII mice with homogenate prepared from dorsal root ganglia (DRG) tissue sampled from three vCJD patients (CJD 38, CJD 39 and CJD 40). The incidence of clinical signs of disease, TSE-specific vacuolar pathology and deposition of PrP in each experimental group are detailed in Table 6.7.

One of the vCJD DRG (CJD 38) transmitted disease readily to RIII mice resulting in a mean incubation period of 528 days. This was similar to the incubation period for vCJD spleen and tonsil tissue using the same mouse line, suggesting similar levels of infectivity in this DRG homogenate to that found in the vCJD lymphoid tissues. The other two DRG homogenates gave evidence of transmission in only a small number of mice, indicating lower levels of infectivity in these homogenates.

Transmission of infectivity from DRG was confirmed by the presence of vacuolar pathology in some RIII mice following experimental challenge with each DRG inoculum (Table 6.7). In general, the appearance of vacuolar pathology in the mouse brain corresponded to the development of clinical TSE in the mice. Sufficient mice showing both clinical and vacuolar signs of TSE disease were available from the experimental challenge of DRG inoculum from CJD 38 for the construction of a lesion profile. The pattern of vacuolar degeneration closely resembles that seen in RIII mice challenged with vCJD lymphoid tissues, specifically with the targeting of vacuolar pathology in the medulla (G1), hypothalamus (G4) and the septum (G7) (Figure 6.9). This suggests that the vCJD agent, present in DRG is similar to that found in lymphoid tissues.

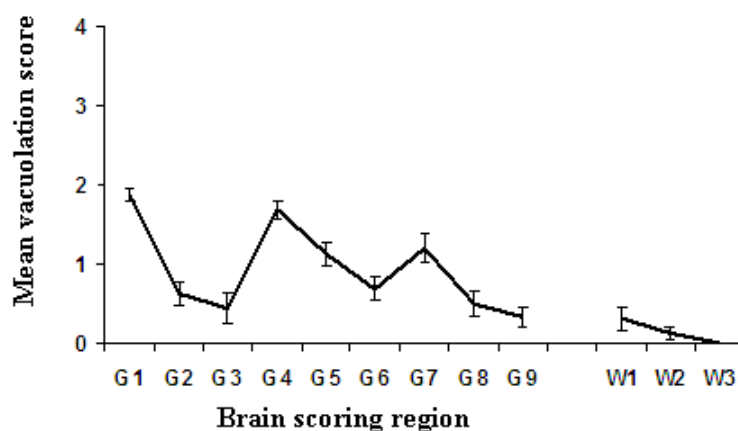
Table 6.7: Primary transmission from vCJD DRG homogenates

Incidence of clinical signs (Clin) of disease and vacuolar degeneration (Vac path) and the accumulation of PrP (PrP path) in RIII mice intracerebrally inoculated with vCJD DRG homogenate. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Mean incubation periods in days are shown in brackets \pm S.E.M.

	Mouse line		
	RIII		
Source	Clin	Vac path	PrP path
CJD 38	17/22 (528 \pm 36)	20/22	nt
CJD 39	2/18 (602, 687)	2/18	2/18
CJD 40	3/18 (611, 678, 856)	3/18	5/18

Figure 6.9: Lesion profiles for mice inoculated with vCJD DRG homogenate

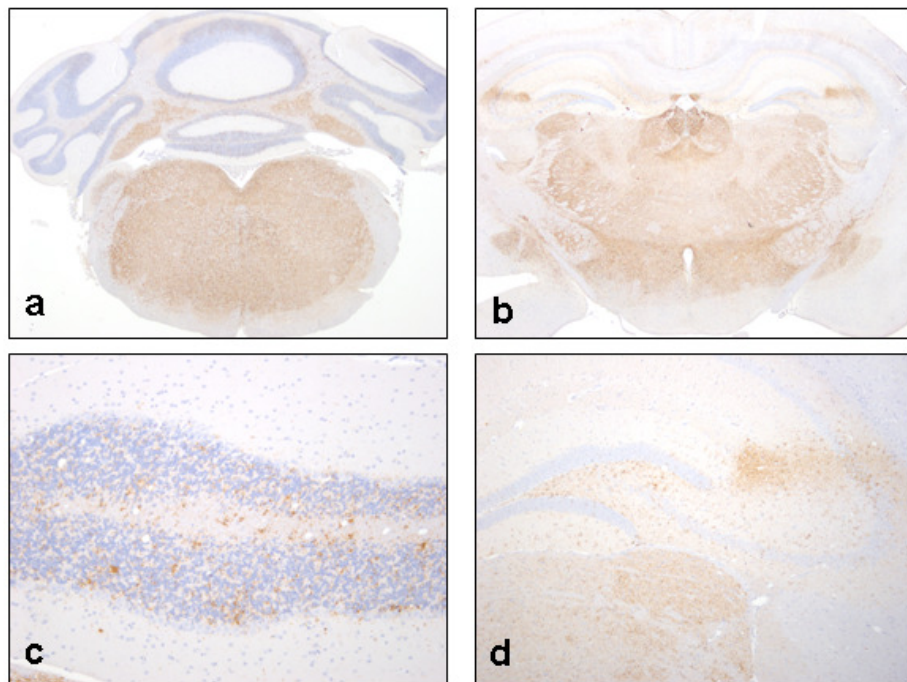
Lesion profiles are constructed from 17 RIII mice scoring positive for both clinical TSE disease and TSE-specific vacuolar pathology following inoculation with DRG homogenate from CJD 38. (G) grey matter scoring regions, (W) white matter scoring regions of the mouse brain. Mean lesion profiles are shown with standard error bars (\pm S.E.M).



Further confirmation of the successful transmission of infectivity from DRG was the observation of PrP in the brains of RIII mice examined immunohistochemically (Table 6.7). Patterns of PrP accumulation in the brain were closely similar in RIII mice inoculated with the different DRG homogenates. Furthermore, the pattern of PrP accumulation was similar to that of mice inoculated with vCJD lymphoid homogenates with a peri-cellular staining pattern for PrP throughout the medulla, the thalamus and hypothalamus regions of the brain (Figure 6.10a and b). PrP accumulation was also observed to a lesser extent in the granular and molecular layer of the cerebellum (Figure 6.10c). In the hippocampal region, PrP staining pattern mirrored that of other vCJD tissue types experimentally inoculated into RIII mice, in which PrP deposition was found to target the CA2 region of the hippocampus (Figure 6.10d).

Figure 6.10: PrP deposition in RIII mice inoculated with vCJD DRG homogenate

Intense granular and peri-cellular deposits of PrP throughout the (a) medulla, (b) thalamus and hypothalamus in an RIII mouse following experimental challenge with a vCJD DRG homogenate. (c) Small granular deposits of PrP within the granular and molecular layer of the cerebellum. (d) Targeting of PrP in the CA2 region of the hippocampus. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x25 (a, b), x100 (d), x200 (c).



6.3.7 Trigeminal ganglia tissue

Three transmission experiments were carried out in RIII mice with homogenate prepared from trigeminal ganglia tissue (TRG) sampled from three vCJD patients (CJD 38, CJD 39 and CJD 40). Details of the incidence of clinical signs of disease, TSE-specific vacuolar pathology and PrP accumulation in each experimental group are shown in Table 6.8.

Two TRG homogenates (CJD 38 and CJD 40) transmitted disease readily in RIII mice with mean incubation periods of 536 and 579 days (Table 6.8). No clinical evidence of TSE disease was seen in the mice inoculated with the remaining TRG homogenate. However, further histological analysis of the brains at post-mortem showed vacuolar pathology in one RIII mouse. This confirmed transmission of disease from this TRG but indicates lower infectivity levels compared to the other two TRG tissue samples. Mean incubation periods for mice inoculated with TRG homogenates were comparable to that seen in transmissions from DRG, suggesting similar levels of infectivity in these two peripheral neural tissues.

The presence of vacuolar pathology in the brain of a proportion of the mice experimentally challenged with all three TRG inocula, confirmed the transmission of a TSE in a proportion of the inoculated mice. Sufficient mice were available from the experimental challenge of DRG homogenate from patients CJD 38 and CJD 40 for the construction of a lesion profile (Figure 6.11). Patterns of vacuolar pathology as represented by the lesion profile were closely similar between the two TRG homogenates, indicating that the mice were infected with the same strain of agent. In addition, the targeting of vacuolar pathology was closely similar to that observed in transmissions from vCJD spleen, tonsil, lymph node and DRG in the same mouse line.

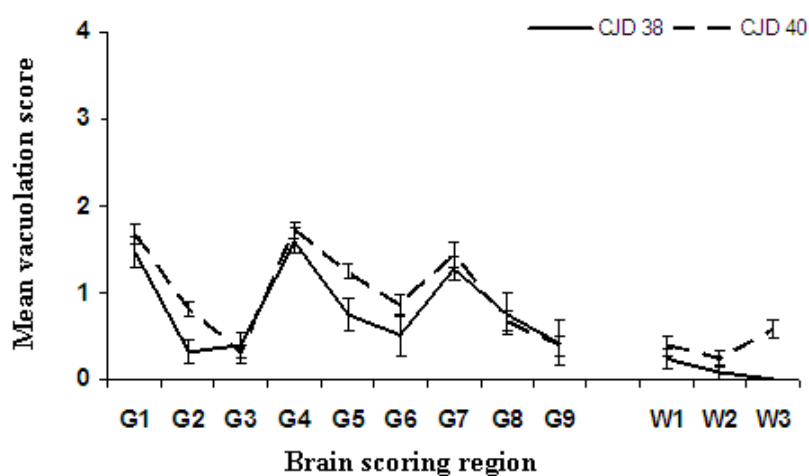
Table 6.8: Primary transmission from vCJD TRG homogenates

Incidence of clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in RIII mice intracerebrally inoculated with vCJD TRG homogenates. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Mean incubation periods in days are shown in brackets \pm S.E.M.

	Mouse line		
	RIII		
Source	Clin	Vac path	PrP path
CJD 38	14/18 (536 \pm 30)	16/18	16/18
CJD 39	0/19	1/19	7/19
CJD 40	21/34 (579 \pm 15)	26/34	18/20

Figure 6.11: Lesion profiles for mice inoculated with vCJD TRG homogenates

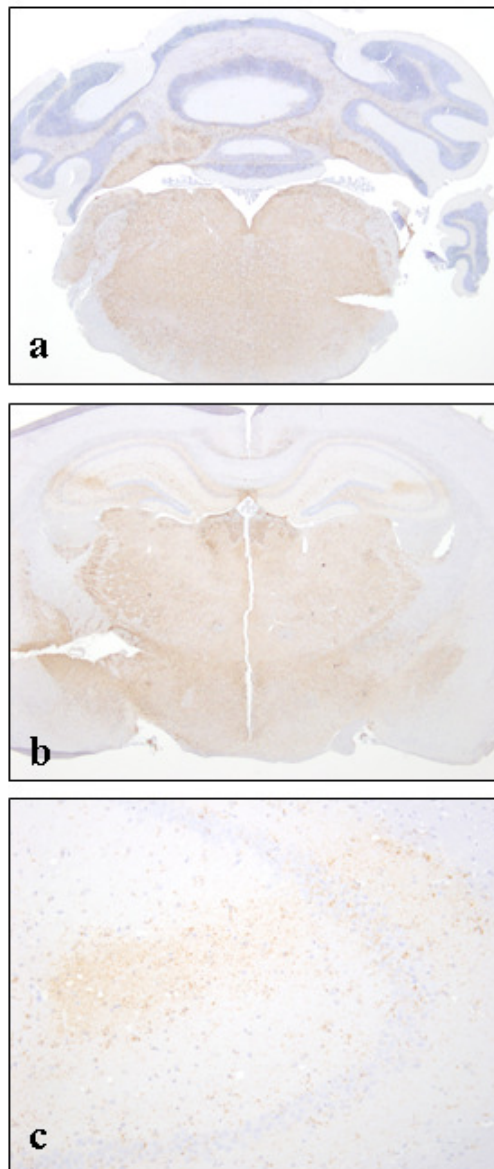
Lesion profiles are constructed from a minimum of five RIII mice scoring positive for both clinical TSE disease and TSE-specific vacuolar pathology. (G) grey matter scoring regions, (W) white matter scoring regions of the mouse brain. Mean lesion profiles are shown with standard error bars (\pm S.E.M).



All inoculated mice with a positive score for clinical signs of disease and vacuolar degeneration in the brain showed PrP accumulation in the brain following immunohistochemical analysis. In addition, PrP accumulation was demonstrated in a proportion of mice that had shown no evidence of any clinical or vacuolar signs of a TSE (Table 6.8). Patterns of PrP accumulation in the brain were closely similar between individual RIII mice with heavy deposits of PrP in the medulla (Figure 6.12a) and throughout the thalamus and hypothalamus (Figure 6.12b) as well as around areas of intense vacuolation. The targeting of PrP in the CA2 region of the hippocampus, a characteristic feature of the different vCJD lymphoid and peripheral neural tissues in RIII mice was also seen following experimental challenge of TRG homogenate (Figure 6.12c). This similarity in the PrP accumulation in the mouse brain is indicative of a similarity in the agent strain between these different tissue types.

Figure 6.12: PrP deposition in RIII mice inoculated with vCJD TRG homogenate

(a) Peri-cellular and granular deposition of PrP in the medulla and cerebellum. **(b)** Widespread intense PrP deposition in the thalamus, hypothalamus and hippocampus following experimental challenge with a vCJD TRG homogenate. **(c)** Targeting of granular PrP deposits in the CA2 region of the hippocampus. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x25 (a, b), x200 (c).



6.3.8 Spinal cord tissue

Tissue homogenates prepared from spinal cord tissue sampled from three vCJD patients (CJD 38, CJD 39 and CJD 40) were experimentally inoculated into RIII mice. Details of the incidence of clinical signs of disease and TSE-specific vacuolar pathology in the inoculated mice, and results from the investigation of PrP accumulation in each experimental group are shown in Table 6.9.

Clinical TSE disease was observed in the majority of mice inoculated with each spinal cord homogenate, with mean incubation periods of 458, 587 and 568 days (Table 6.9). Experimental challenge of the spinal cord sample from CJD 38 provided the shortest incubation period of any of the vCJD lymphoid and peripheral neural tissues examined, indicating the highest levels of infectivity in this homogenate. However, with an incubation period of 458 days, this was still longer than previous transmissions from vCJD brain tissue (Chapter 5, section 5.3.1), indicating that levels of infectivity are lower than those found in brain tissue. The 100-day incubation period difference found between the spinal cord inoculum from CJD 38 and CJD 39 and CJD 40 is more likely to be attributed to variations in levels of infectivity between the different spinal cord homogenates

TSE-specific vacuolar pathology in the majority of the inoculated mice confirmed the clinical diagnosis of a TSE. Furthermore, sufficient mice were available from all experimental groups for the construction of a lesion profile. Patterns of vacuolar pathology were remarkably similar for each spinal cord homogenate, evidence that the same strain of agent is present in each vCJD spinal cord (Figure 6.13) and further evidence that incubation period differences are due to variations in levels of infectivity. The targeting of vacuolar pathology in the RIII mice was similar to that described in the previous transmissions from vCJD lymphoid tissues and peripheral neural tissues with the most severe vacuolar pathology being found in the medulla, hypothalamus and the septum (Figure 6.13).

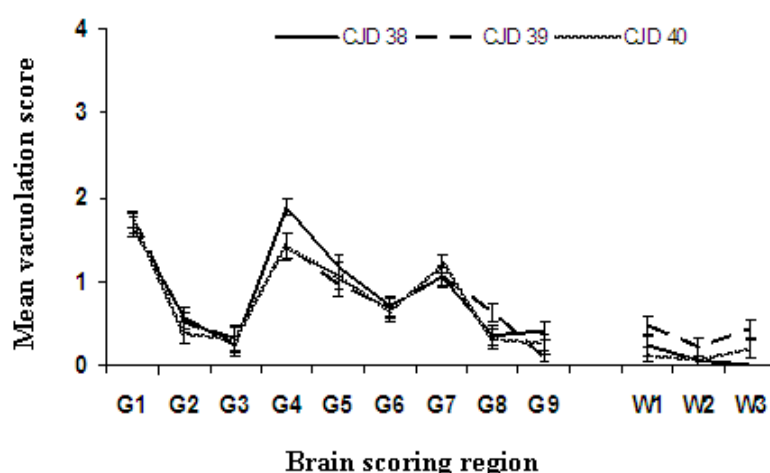
Table 6.9: Primary transmission from vCJD spinal cord homogenates

Clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in RIII mice intracerebrally inoculated with vCJD spinal cord homogenates. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. Mean incubation periods in days are shown in brackets \pm S.E.M

	Mouse line		
	RIII		
Source	Clin	Vac path	PrP path
CJD 38	17/20 (458 \pm 13)	18/20	nt
CJD 39	19/23 (587 \pm 20)	19/23	7/7
CJD 40	16/21 (568 \pm 16)	16/21	5/5

Figure 6.13: Lesion profiles for mice inoculated with vCJD spinal cord homogenates

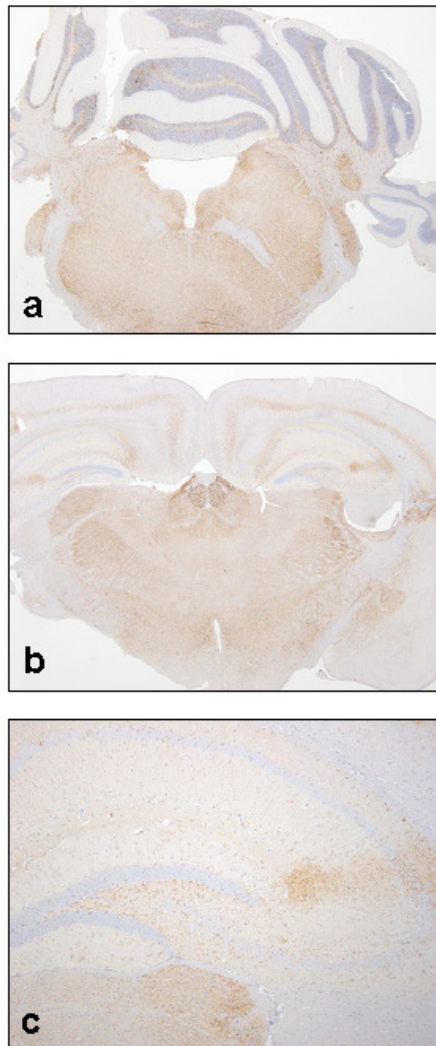
Lesion profiles are constructed from RIII mice scoring positive for both clinical TSE disease and TSE-specific vacuolar pathology. (G) grey matter scoring regions, (W) white matter scoring regions of the mouse brain. Mean lesion profiles are shown with standard error bars (\pm S.E.M).



As expected, inoculation of spinal cord homogenate produced an intense pattern of PrP deposition in the brain. The pattern of PrP accumulation was similar between mice inoculated with the different spinal cord homogenates and closely resembled those of all other vCJD tissue homogenates in the same mouse line. A widespread distribution of peri-cellular and granular PrP deposits throughout the mouse brain was observed (Figure 6.14). As described with other vCJD tissue homogenates, the most intense deposition of PrP was found in the medulla oblongata (Figure 6.14a), the thalamus and hypothalamus and around areas of extensive vacuolar pathology (Figure 6.14b). The targeting of PrP in the CA2 region of the hippocampus in the mice (Figure 6.14c) further confirms the similarity in the transmission properties of RIII mice inoculated with spinal cord tissue to that described from challenge with other lymphoid and peripheral neural tissues.

Figure 6.14: PrP deposition in RIII mice inoculated with vCJD spinal cord homogenate

(a) Heavy peri-cellular and granular deposition of PrP in the medulla and cerebellum of an RIII mouse. (b) Widespread intense PrP deposition in the thalamus, hypothalamus, hippocampus and cerebral cortex following experimental challenge with a vCJD spinal cord homogenate. (c) Targeting of granular PrP deposits in the CA2 region of the hippocampus. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin. Original magnification; x25 (a, b), x100 (c)



6.3.9 Bone Marrow

Experimental transmission of bone marrow sampled from three vCJD patients (CJD 38, CJD 39 and CJD 40) was carried out in RIII mice. Details of the incidence of clinical signs of disease, TSE-specific vacuolar pathology and deposition of PrP in the mouse brain in each experimental group are shown in Table 6.10.

Only one RIII mouse inoculated with vCJD bone marrow received a positive score for the development of clinical signs of a TSE (Table 6.10); however, histological analysis failed to confirm a TSE in this mouse with no evidence of vacuolar degeneration in the brain. In the absence of any TSE-specific vacuolar pathology in these mice, lesion profiling was not a relevant strain typing parameter in the experimental challenge of vCJD bone marrow. The lack of any vacuolar pathology in the mice therefore provides no evidence for the transmission of TSE infectivity from vCJD bone marrow. Furthermore, immunohistochemical examination for the prion protein on all inoculated mice also failed to show any evidence of transmission from vCJD bone marrow (Table 6.10).

Table 6.10: Primary transmission from vCJD bone marrow samples

Incidence of clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in RIII mice intracerebrally inoculated with vCJD bone marrow samples. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Mouse line		
	RIII		
Source	Clin	Vac path	PrP path
CJD 38	0/20	0/20	0/20
CJD 39	1/20	0/20	0/20
CJD 40	0/21	0/21	0/21

6.3.10 Blood components

Inoculation of buffy coat and plasma separated from blood samples taken from four sCJD and four vCJD patients were carried out in RIII and VM mice. Details of the incidence of clinical signs of disease, TSE-specific vacuolar pathology and results from the deposition of PrP in the mouse brain in experimental groups are shown in Table 6.11.

Clinical signs of disease were observed in a proportion of VM and RIII mice following inoculation with plasma (15/245 mice) and buffy coat (3/276 mice) samples from sCJD and vCJD patients (Table 6.11). Histological analysis failed to confirm a TSE in any of these mice, with no evidence of vacuolar degeneration in the brain. As discussed in Chapter 4, section 4.3.1.1, it is the working assumption of this thesis that the neuropathological assessment of these 18 mice that is definitive and the positive clinical signs are either in doubt or indicative of an unrelated illness or ageing.

However, TSE-specific vacuolar pathology was observed in one VM mouse from the group experimentally challenged with a sCJD buffy coat sample from a patient who was homozygous for methionine at codon 129 and known to have a mixed PrP^{res} type in the brain (patient CJD 11). No evidence of transmission, as judged by the development of vacuolar pathology, was observed in any mouse (RIII or VM) after challenge with vCJD buffy coat (Table 6.11). In the absence of sufficient mice available for the construction of a lesion profile it was not possible to compare the single VM mouse profile with the lesion profiles from other peripheral neural and lymphoid tissues. In contrast, no evidence of vacuolar pathology was found in any mouse after challenge with CJD (sCJD or vCJD) plasma samples. This suggests that either transmission of a TSE does not occur from this blood fraction or that the titres of infectivity are lower than that associated with the buffy coat fraction. In summary the lesion profile is not a relevant parameter in the strain typing of buffy coat or plasma from CJD patients in this study.

Immunohistochemical analysis for the prion protein was carried out on all mice (VM and RIII) inoculated with buffy coat and plasma components from each sCJD and vCJD patient. Only one mouse showed any evidence of PrP deposition in the brain (Table 6.11). This same VM mouse had shown TSE-specific vacuolar degeneration in the brain, thus confirming that this mouse was positive for TSE pathology after inoculation with this probable sCJD MM 1+2 sCJD buffy coat fraction. The pattern of PrP deposition in this mouse was consistent with that observed in VM mice challenged with sCJD brain homogenate described in chapter 4, characterised by a restricted PrP pathology targeting the MHb (Figure 6.15a). In addition, some light granular deposition of PrP was observed in the superior colliculus (Figure 6.15b) in which vacuolar pathology is a consistent feature of mice following inoculation with sCJD brain homogenates (chapter 4) and in the pons (Figure 6.15c), a region of the brain particularly targeted in mice challenged with the MM2 subtype of sCJD.

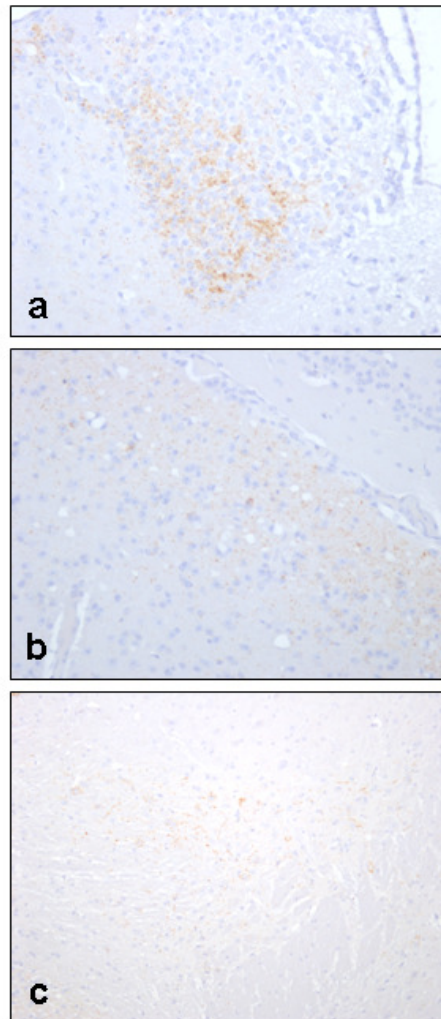
Table 6.11: Primary transmission from vCJD blood components

Incidence of clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in RIII and VM mice intracerebrally inoculated with buffy coat and plasma fractions taken vCJD and sCJD patients. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

			Mouse line					
			RIII			VM		
Agent	Source	Inoculum	Clin	Vac path	PrP path	Clin	Vac path	PrP path
sCJD	CJD 10	Buffy coat	0/13	0/13	0/13	0/15	0/15	0/15
	CJD 11	Buffy coat	0/22	0/22	0/22	0/20	1/20	1/20
		Plasma	0/21	0/21	0/21	0/22	0/22	0/22
	CJD 15	Buffy coat	0/17	0/17	0/17	0/14	0/14	0/14
	CJD 18	Buffy coat	0/12	0/12	0/12	0/14	0/14	0/14
		Plasma	0/20	0/20	0/20	1/20	0/20	0/20
vCJD	CJD 31	Buffy coat	0/18	0/18	0/18	0/21	0/21	0/21
		Plasma	0/23	0/23	0/23	0/21	0/21	0/21
	CJD 35	Buffy coat	1/17	0/17	0/17	0/14	0/14	0/14
		Plasma	0/18	0/18	0/18	0/23	0/23	0/23
	CJD 36	Buffy coat	0/20	0/20	0/20	1/28	0/28	0/28
		Plasma	8/27	0/27	0/27	2/19	0/19	0/19
	CJD 37	Buffy coat	0/14	0/14	0/14	1/18	0/18	0/18
		Plasma	4/14	0/14	0/14	0/17	0/17	0/17

Figure 6.15: PrP deposition in a VM mouse challenged with sCJD buffy coat

Faint granular deposition of PrP in the **(a)** medial habenular nucleus (MHb) and **(b)** superior colliculus following inoculation with a sCJD buffy coat sample. **(c)** Targeting of faint peri-cellular PrP deposits around in pons in the same mouse brain. Sections shown were labelled with the anti-PrP antibody 6H4 in combination with the CSA detection method, visualised with DAB and counterstained with haematoxylin Original magnification; x200 (d), x400 (a and b)



6.3.11 Cerebrospinal Fluid

Inoculation of CSF samples from two vCJD patients was carried out in RIII mice. Details of the incidence of clinical signs of disease, TSE-specific vacuolar pathology and deposition of PrP in the mouse brain in each experimental group are shown in Table 6.12.

Clinical signs were observed in six mice inoculated with CSF from a single vCJD patient (Table 6.12). However, histological analysis of all inoculated mice, failed to confirm a TSE in any mouse challenged, with no evidence of vacuolar degeneration in the brain. As described previously (Chapter 4, section 4.3.1.1), the neuropathological assessment is considered definitive in such cases. Furthermore, immunohistochemical analysis for the prion protein showed no evidence of PrP accumulation within the brain of inoculated mice. Therefore, there is no evidence of transmission of infectivity from CSF in this thesis.

Table 6.12: Primary transmission from vCJD CSF samples

Incidence of clinical signs (Clin), vacuolar degeneration (Vac path) and PrP deposition (PrP path) in RIII intracerebrally inoculated with vCJD CSF samples. Mice sacrificed with intercurrent disease before 250 dpi were excluded from the analysis. In mice with a positive score for clinical signs of a TSE and a negative score for vacuolar pathology, the neuropathological assessment is considered definitive.

	Mouse line		
	RIII		
Source	Clin	Vac path	PrP path
CJD 38	6/21	0/21	0/21
CJD 40	0/20	0/20	0/20

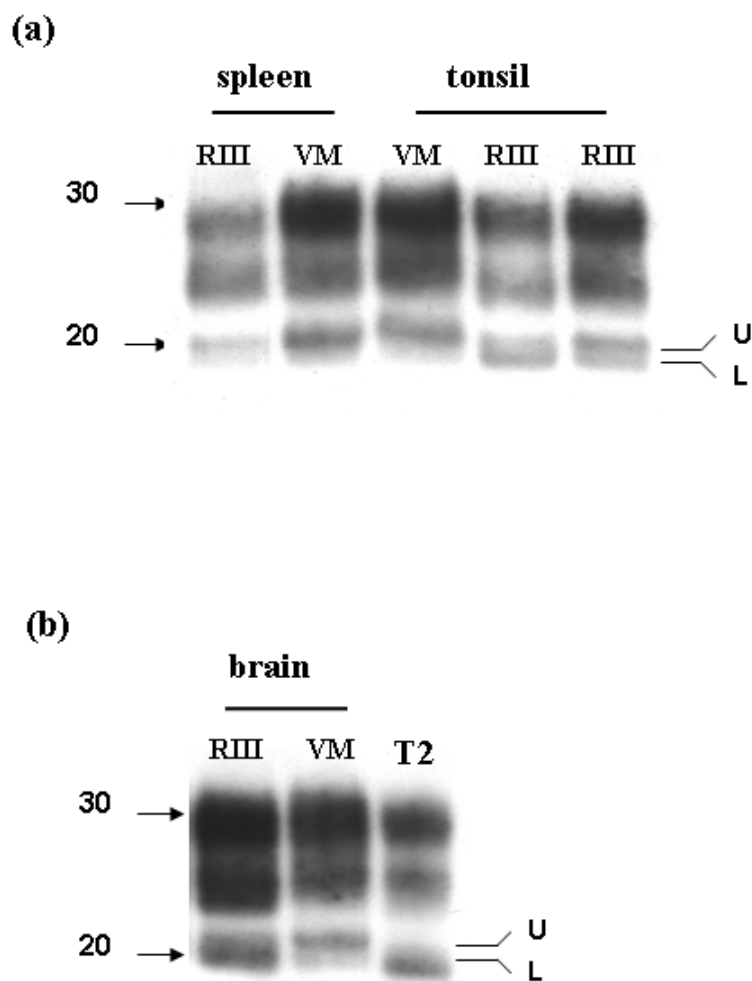
6.3.12 Biochemical isotyping of PK resistant PrP^{Sc}

Frozen tissues available for the investigation of PrP^{res} type in mice following inoculation with non-CNS tissue homogenates were limited. Only three RIII mice (two inoculated with vCJD tonsil tissue; one inoculated with vCJD spleen tissue) and two VM mice (one inoculated with vCJD tonsil tissue; one inoculated with vCJD spleen tissue) had frozen CNS tissue available for testing. Each mouse had received a positive score for clinical signs of disease and vacuolar pathology in the brain following inoculation with the different tissue types. Characterisation of the PrP^{res} type in the mouse brain was carried out using a standard Western blot analysis method used in the diagnostic typing of human TSEs (Chapter 2, section 2.7). Western blot analysis was carried out on 5µl extracts of a 10% brain tissue weight to buffer volume (w/v).

Overall, transmission from vCJD spleen and tonsil in RIII and VM mice demonstrated a glycoform pattern and electrophoretic mobility closely resembling that observed in the same mouse lines following transmission from vCJD brain tissue (Figure 6.16). However, the one RIII mouse inoculated with vCJD spleen homogenate that was available for analysis had a substantially modified glycoform ratio with an observable loss of the predominance of the diglycosylated fragment of the protein (Figure 6.16a). The appearance of the unglycosylated fragment as a doublet composed of distinct upper and lower bands, a feature observed in the mice experimentally infected with vCJD brain homogenate (Chapter 5, section 5.3.5), was also a feature of these five mice. There did not appear to be a correlation between the different mouse *Prn-p* genotypes and the relative abundance of the upper and lower bands comprising the unglycosylated doublet.

Figure 6.16: Western blot analysis of PrP^{res} in mice inoculated with vCJD tonsil and spleen

Western blot analysis of PrP^{res} in CNS samples of RIII and VM mice after experimental transmission of vCJD **(a)** spleen and tonsil homogenate and **(b)** brain homogenate. Tissues are analysed after digestion with proteinase K. Molecular weight markers are shown in kDa. (T2) human vCJD diagnostic standard; (U) upper band of the unglycosylated fragment; (L) lower band of the unglycosylated fragment



6.4 Results summary

A full summary of the results examined in this Chapter is presented in Table 6.13. Infectivity was detectable to some extent in vCJD spleen, tonsil, cervical lymph node, appendix, spinal cord, peripheral nerve, DRG and TRG. No evidence of a TSE was observed in mice inoculated with vCJD bone marrow, plasma or CSF samples. In addition, no evidence of infectivity was found in sCJD spleen and plasma samples. However, infectivity was demonstrated to some degree in a sCJD buffy coat sample. Differences and similarities were observed in the transmission properties of the various vCJD peripheral in wild-type mice and were characterised by the following features;

- In general, vCJD-infected mice develop a progressive clinical disease. However, preclinical infection was detected in mice inoculated with vCJD appendix and peripheral nerve isolates.
- Mean incubation periods are extended in mice inoculated with lymphoid and peripheral neural tissues compared to those observed in the same mouse line challenged with vCJD brain homogenate (Figure 6.17).
- Incubation periods in mice vary between different tissue inocula and also between the same tissue inocula from different vCJD cases. This suggests variation in the levels of infectivity between different tissue types and between the same tissue types sampled from different vCJD patients (Figure 6.17).
- Lesion profiles show a remarkable similarity (most obvious in the RIII line) between different tissue types with a pattern which is closely similar to that observed in the same mouse lines following challenge with vCJD brain homogenate. (Figure 6.18).
- Lesion profiles demonstrate a different pattern of vacuolar degeneration between RIII and VM mice (Figure 6.18).
- Patterns of PrP deposition in vCJD-infected mice are remarkably similar following inoculation with different tissue types and a pattern that mirrors that observed in mice following challenge with vCJD brain homogenate.
- Amyloid plaques are not a feature of vCJD in wild-type mice following inoculation with peripheral tissues.

Overall, results indicate similar transmission properties in mice challenged with different vCJD tissue types. These transmission properties are similar to those found with vCJD brain homogenate described in chapter 5. This indicates that the same strain of agent is present in all vCJD-infected tissues examined in this thesis.

Table 6.13: Summary of experimental transmissions

Positive (+) and negative (-) scores for the presence of clinical disease (Clin), vacuolar pathology (Vac path) and PrP deposition (PrP path) in mice following inoculation with different tissue homogenates. Positive scores are based on results from at least one transmission experiment.

	Tissue	Clin	Vac path	PrP path
vCJD	spleen	+ve	+ve	+ve
	tonsil	+ve	+ve	+ve
	lymph node	+ve	+ve	+ve
	appendix	-ve	-ve	+ve
	peripheral nerve	*+ve	*+ve	+ve
	spinal cord	+ve	+ve	+ve
	DRG	+ve	+ve	+ve
	TRG	+ve	+ve	+ve
	bone marrow	-ve	-ve	-ve
	buffy coat	-ve	-ve	-ve
	plasma	-ve	-ve	-ve
	CSF	-ve	-ve	-ve
sCJD	spleen	-ve	-ve	-ve
	buffy coat	-ve	*+ve	*+ve
	plasma	-ve	-ve	-ve

* Evidence for transmission based on one mouse

Figure 6.17: Mean incubation times in vCJD challenged mice

Mean incubation periods in RIII and VM mice after experimental challenge with brain homogenate and different tissue homogenates from vCJD patients. Data are mean \pm S.E.M. (FC) frontal cortex, (CB) cerebellar cortex, (IC) intracerebral inoculation, (IP) intraperitoneal inoculation.

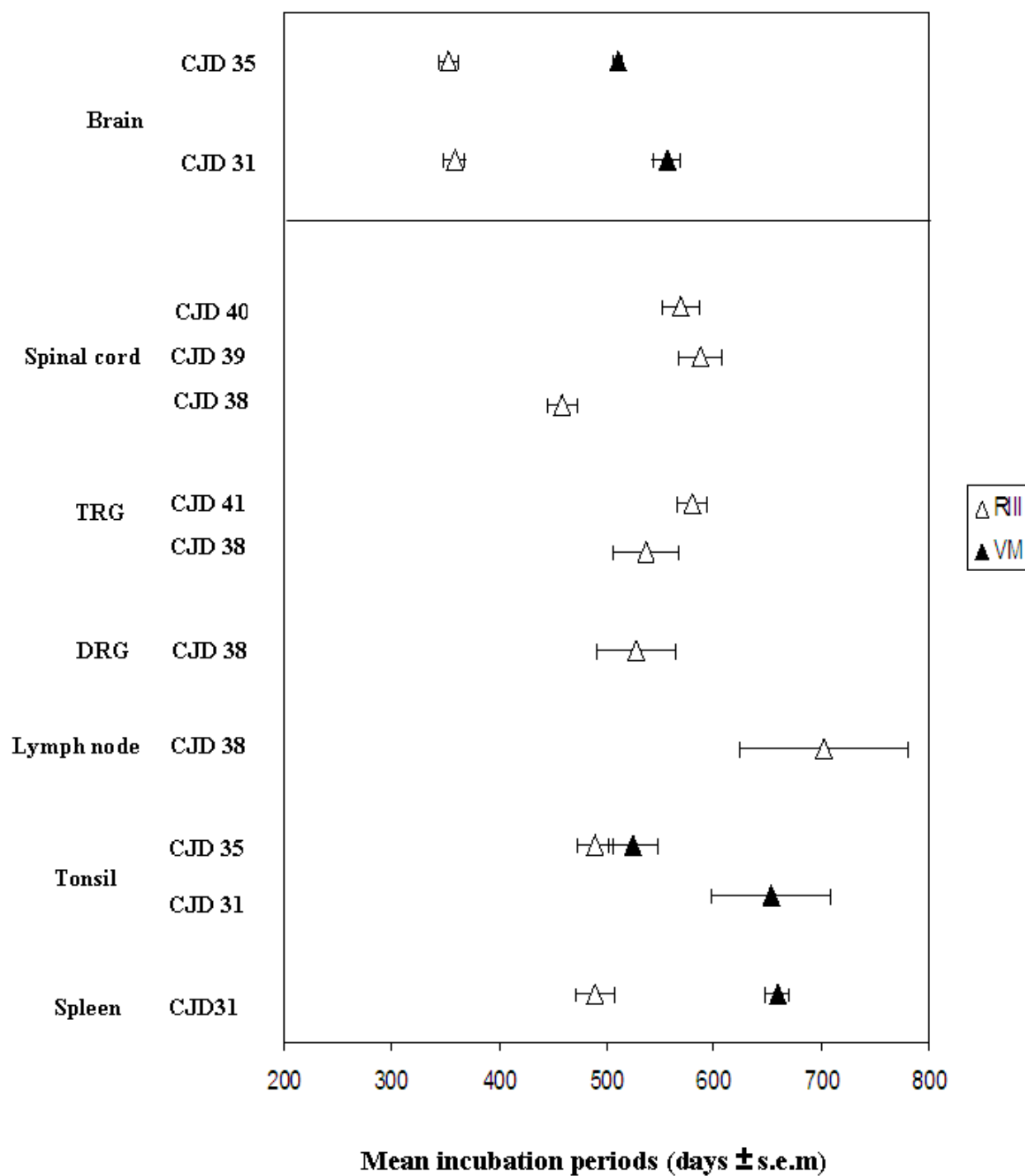
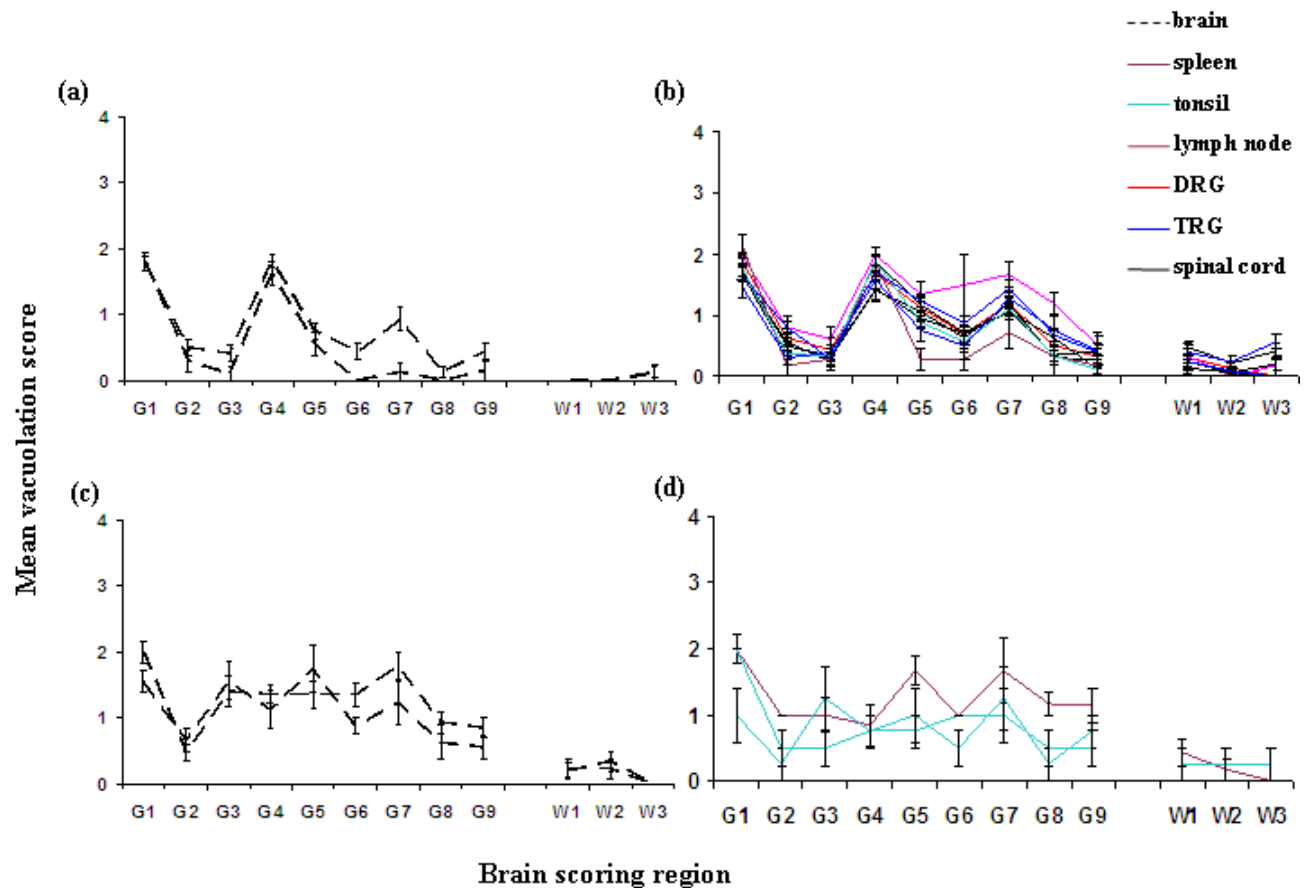


Figure 6.18: Lesion profiles for vCJD inoculated mice

Comparison of lesion profiles in RIII (a, b) and VM mice (c, d) after experimental challenge with vCJD brain homogenate (a, c) and lymphoid and neural tissue homogenates (b, d). Lesion profiles are constructed from a minimum of five mice scoring positive for both clinical TSE disease and TSE-specific vacuolar pathology. (G) grey matter scoring regions, (W) white matter scoring regions of the mouse brain. Mean lesion profiles are shown with standard error bars (\pm S.E.M).



6.5 Discussion of experimental results

This chapter had two specific aims. Firstly to assess whether infectivity is present within specified peripheral neural and lymphoid tissues of patients with vCJD and sCJD; and secondly to characterise the agent strain present within these peripheral tissues. Data presented in this chapter confirms the original demonstration of infectivity in spleen and tonsil (Bruce et al., 2001) and extends the evidence of infectivity to other tissues of CJD patients in a number of important respects. Firstly, this study extends the evidence for a widespread infection of the lymphoid system at the end-stage of disease with infectivity demonstrated in vCJD appendix and cervical lymph node. Secondly, the data provides the first direct demonstration of infectivity in the peripheral nervous system of vCJD patients. Thirdly, the identification of TSE pathology in a single mouse after challenge with a sCJD buffy coat sample suggests the possibility of infection in sCJD blood, and finally, this study confirms the same strain of agent within vCJD lymphoid and neural tissues.

6.5.1 Infectivity levels within vCJD peripheral neural and lymphoid tissues

In general, infectivity was detected in tissues from CJD patients in which the presence of the abnormal prion protein has been previously reported. Clinical and pathological signs associated with a TSE were observed to some extent from vCJD spleen, tonsil, lymph node, appendix, DRG, TRG, peripheral nerve, spinal cord and sCJD buffy coat. Previous transmission experiments have shown that titres of infectivity in vCJD spleen and tonsil are lower than in brain resulting in extended incubation periods (Bruce et al., 2001). Overall, analysis of incubation period data in this study confirms these findings and indicates that levels of infectivity in other vCJD lymphoid tissues and neural tissues are also lower than those demonstrated in brain (Figure 6.17). In tissue homogenates that transmitted disease readily to mice resulting in clinical disease (vCJD spleen, tonsil, lymph node, DRG, TRG and spinal cord), incubation periods were broadly similar irrespective of the tissue type used in the inoculation (Figure 6.17). This suggests that levels of infectivity are similar within these different inocula and are comparable to those infectivity titres previously demonstrated in vCJD spleen and tonsil (Bruce et al., 2001). However, efficiency of transmission from each tissue type varied between vCJD patients. In the

transmission of peripheral neural tissue, one vCJD DRG and two TRG transmitted disease readily to mice with incubations comparable to those seen in spleen and tonsil. However the other DRG and TRG homogenates gave evidence of transmission in only a small number of the mice. The results from the lymphoid tissues also varied between patients. One of the vCJD lymph nodes gave definite transmission to mice; although the long incubation periods and partial take suggest only low infectivity levels. Another vCJD lymph node sample showed only trace infectivity levels and the third was completely negative (based on clinical and pathological assessment). This suggests there may be patient-to-patient variation in levels of infectivity.

However, is variation in infectious titre the only possible explanation for the differences found in the efficiency of transmission between the different lymphoid tissues? Accumulations of pathological PrP have been detected in appendix samples surgically removed from patients that went on to develop clinical vCJD eight months or two years later (Hilton et al., 2004a), suggesting a long-term lymphoid tissue involvement. However, data from this study demonstrates that transmission from appendix tissue is relatively inefficient compared to spleen, tonsil and lymph node. Furthermore, immunohistochemistry confirmed the deposition of PrP in these appendix tissues, so it is important to ask why were there so few mice showing evidence of transmission compared to the other lymphoid tissues. This could simply be a sampling problem, as was discussed earlier in Chapter 3. It may be that infectivity was present in the appendix tissue, but that the small tissue samples prepared as inoculum had missed the major infectivity foci in the lymphoid follicles, perhaps because of fibrosis or previous inflammation and scarring in the appendixes. Lymphoid follicles containing germinal centres are irregularly distributed in the adult appendix; this was confirmed on immunohistochemistry for PrP in the three appendixes sampled in this study, which showed positively staining germinal centres in a patchy distribution, consistent with an earlier study (Head et al., 2004b). Furthermore, Western blot analysis of 200µl extracts of residual inoculum from two of the inoculations (CJD 38 and CJD 40) failed to show any detectable levels of PrP^{res}, supporting evidence that the appendix tissue sampled for these inoculations

may have missed the major foci of infectivity. It could be that the differences observed in the efficiency of transmission between other tissue types in this study may also reflect sampling difficulties in tissues in which the deposition of PrP^{Sc} is known to be much more heterogeneous than those found in brain, (Joiner et al., 2002; Hilton et al., 2004a; Head et al., 2004b), rather than a reflection of differences in levels of infectivity in the tissues as a whole.

6.5.2 The same agent strain is present within different vCJD peripheral lymphoid and neural tissues

Comparison of the transmission properties from the inoculation of tissue samples from vCJD spleen, tonsil, cervical lymph node, appendix, peripheral nerve, DRG, TRG and spinal cord has provided convincing evidence that the same strain of agent is present within these different tissues. Overall, lesion profiling in RIII mice showed a remarkably similar pattern for each vCJD inoculation, regardless of the tissue type used to prepare the inoculum (Figure 6.18). In addition, the different vCJD tissues produced a consistent pattern and targeting of PrP pathology in the RIII mouse brain. These findings confirm those of previous studies which report that the strain properties of the agent are not substantially altered by the tissue in which it replicates (Beringue et al., 2008). This is interesting because minor differences in the PrP^{res} glycosylation and fragment size, factors proposed to be important in enciphering strain-specific information, have been described in vCJD lymphoreticular and peripheral neural tissues compared to brain although they all retain a resemblance to PrP^{res} in vCJD brain (Head et al., 2004b). The lack of any frozen CNS tissue in the majority of the inoculations has meant that establishing whether these tissue or cell-specific influences on the conformation or glycosylation of PrP are conserved on transmission to mice is largely unanswered in this study. However, analysis of a limited number of RIII and VM mice following inoculation with vCJD spleen and tonsil tissue generally produced a glycoform pattern closely resembling that of vCJD brain and in murine CNS after experimental challenge with vCJD brain homogenate (chapter 5). This provides further evidence that the same strain of agent is present in vCJD tissues examined in this study and that the differences seen in PrP^{res} type

between brain and other tissue types in vCJD are not fundamental properties of the agent and may be tissue specific epiphenomena.

6.5.3 Comparison of infectivity levels in sCJD and vCJD tissues

Direct comparison of infectivity in vCJD and sCJD tissues was limited to spleen and blood fractions (buffy coat and plasma) in this study. The absence of any evidence of transmission from sCJD spleen homogenate supports previous findings that there is a more limited involvement of lymphoid tissues in sCJD in comparison to vCJD (Head et al., 2004b) and suggests a greater potential for iatrogenic spread for vCJD than for sCJD. However this did not appear to hold true when infectivity in different blood components from CJD patients was examined.

The successful transmission of a TSE in sheep after receiving a transfusion of either whole blood or buffy coat fractions from sheep incubating scrapie and experimental BSE demonstrated that infectivity was present in blood (Hunter et al., 2002). Furthermore, the probable transmission of vCJD infectivity in five individuals who received a blood transfusion or blood products from blood donated from asymptomatic vCJD patients confirmed that infectivity was also present within blood in vCJD patients. These findings suggest that blood transfusion may be a highly efficient route of transmission. However, this thesis has shown no evidence of transmission from either vCJD blood fractions (buffy coat and plasma). This may not be a surprising result: it may be that the infectious titres in the blood components, combined with the relatively low volumes inoculated (20µl) were insufficient to cause disease in these mice. It could also be an indication that the intravenous route is more efficient in the transmission of infectivity from blood. Somewhat surprisingly, this study suggests the possibility of infectivity in sCJD blood. With such an anomalous result there are two possible explanations. Firstly, that this is a true result and provides evidence of infectivity in sCJD blood; or that this results from an artefact or some laboratory error. The theoretical considerations of these two possibilities are discussed in full in Chapter 7, section 7.2.9.

It is obviously not possible in humans to trace the accumulation of vCJD infectivity in peripheral tissues through the long incubation period. However, experimental animal studies of other TSEs have shown that infectivity may accumulate rapidly in lymphoid tissues soon after exposure (Kimberlin and Walker, 1979; Kimberlin and Walker, 1989b) and may remain at plateau levels for long periods before the animal develops clinical signs of disease (Dickinson and Fraser, 1969; Dickinson, 1976). Similarly there is evidence of an early involvement of the peripheral nervous system in experimental animal models (Groschup et al., 1999; Beekes and McBride, 2000). If the pathogenesis of disease in vCJD is similar to that seen in these models, an infected asymptomatic individual could represent a transmission risk over a period of years. The data presented in this chapter provides further valuable evidence pertinent to the ongoing risk assessment of the dangers associated with the different neural and peripheral tissues from patients with variant and sporadic CJD.

6.6 Chapter conclusion

This study has extended the evidence for widespread lymphoid tissue infection in vCJD. In addition, inoculations from neural tissues provide the first direct demonstration of infectivity in the peripheral nervous system. Characterisation of the agent strain confirms a single agent strain associated with different vCJD tissues which is closely similar to that observed in vCJD brain. These results provide valuable information for assessing risks of iatrogenic transmission associated with different surgical and medical procedures.

Chapter 7: Discussion

7.1 Overview of the aims of the thesis

The aims of this thesis were to characterise the sCJD and vCJD agent strain by primary transmission and following the subsequent mouse-to-mouse passage of CNS tissue in wild-type mice. In addition, inoculation of the same mouse lines with tissue homogenates prepared from a wide range of peripheral tissues and biological fluids from CJD patients were carried out to investigate the distribution of infectivity and characterise the agent present in tissues other than brain. The principal findings from the data generated in this thesis are itemised below and then discussed in relation to each other and to issues which have arisen within the TSE field during the course of this work.

7.2 Principle findings

- Data from this thesis provides evidence of at least three distinct strains of agent associated with sCJD cases. Two sCJD strains were distinguished by subtle differences in their biological and biochemical properties on transmission to wild-type mice and a third is proposed based on the lack of transmission in the mice.
- Experimental transmission of vCJD in wild-type mice confirms that vCJD appears as a single strain of agent on primary transmission and is distinct from sCJD.
- Data from this thesis extends the evidence for a widespread peripheral lymphoid tissue involvement in vCJD and provides the first direct demonstration of the presence of vCJD infectivity in the peripheral nervous system and evidence of sCJD infectivity in blood.
- Furthermore, data generated shows that there are no tissue-specific adaptations of the vCJD agent.

7.2.1 The relationship between disease phenotype, *PRNP* codon 129 genotype, PrP^{res} type and agent strain in sporadic CJD

The different phenotypic subtypes identified in cases of sCJD (Parchi et al., 1999) are proposed to relate to the propagation of unique strains of sCJD; however, this proposal remains unconfirmed. This thesis represents the first systematic analysis of each of the major sCJD subgroups (classified according to *PRNP* codon 129 genotype and PrP^{res} type combination) based on the “gold standard” strain typing of TSEs in panels of wild-type mice. Three major groupings of sCJD have been distinguished by this method (MM1 and MV1; MM2; MV2, VV1 and VV2), defined by similarities and differences in their transmission properties in the mice. Evidence in support of these three major groups of sCJD was reported in a comparable study examining the survival times and disease phenotype in bank voles experimentally challenged with sCJD subgroups (Nonno et al., 2006). In agreement with the findings in the wild-type mice, voles were most susceptible to infection with brain homogenate from sCJD MM1, MV1 and MM2 cases. The authors inferred the existence of two sCJD strains; one associated with the sCJD MM1 and MV1 subgroups and a second associated with the sCJD MM2 subgroup, based on differences in the clinical disease, neuropathology and biochemical phenotypes observed after primary and secondary transmission. Furthermore, the lack of any evidence of transmission in the voles following inoculation with tissue from sCJD MV2 and VV2 cases was used as evidence that these could represent a further sCJD strain. This is consistent with the findings in the wild-type mice described in this thesis. The sCJD VV1 subgroups was not investigated by the inoculation of bank voles (Nonno et al., 2006).

Both in the bank vole study and in the mouse study described in this thesis, susceptibility to disease appears to be attributable in part to the effect of methionine, rather than valine at *PRNP* codon 129 in the sCJD donor. Only in exceptional cases was transmission observed from VV donors. The numbers of comparative studies in wild-type mice is surprisingly limited; however, in one study examining the transmission properties of Japanese sCJD cases from each of the codon 129 genotypes, a similar ranking of susceptibility of the mice to sCJD cases of each

codon 129 genotype was reported (Tateishi et al., 1996); further evidence that the presence of at least one methionine allele at codon 129 appears to facilitate the transmission of sCJD in wild-type mice. However, it is worth noting that the lack of transmission from the sCJD MV2 inocula in this thesis suggests that susceptibility to disease is not solely a consequence of *PRNP* codon 129 genotype. This will be discussed in detail later in this Chapter.

A potential limitation to the use of wild-type mice in the investigation of human TSEs is the effect of host codon 129 genotype on disease susceptibility. Wild-type mouse lines are all homozygous for methionine at the corresponding *Prn-p* codon 128. So the question of whether the different sCJD transmission properties described in this thesis are a direct result of the presence of the methionine allele at codon 129 in the sCJD donor or merely a consequence of the compatibility between the corresponding PrP genotype of the donor and host species can not be determined. The same question is also relevant in the interpretation of data from the sCJD transmissions to bank voles discussed earlier in this section (Nonno et al., 2006). As in mice, the vole prion protein gene encodes for methionine at the equivalent position to human codon 129; therefore, the question of whether the transmission properties observed in this animal model are a reflection of the compatibility between the donor PrP^{Sc} and recipient PrP^C is also unresolved. Nevertheless, bioassay in wild-type mice distinguishes the MM1, MV1, MM2 cases from the MV2, VV1 and VV2 cases based on the manifestation of clinical disease, neuropathology and the demonstration of PrP^{Sc} in the brain.

In principle it is possible that differences in the efficiency of transmission between sCJD subgroups may simply be due to differences in infectious titres between the different groups rather than a reflection of any strain-specific properties. The lack of transmission from the MV2, VV1 and VV2 subgroups might therefore be taken to mean that these subgroups have a consistently lower titre than the MM1, MV1 and MM2 subgroups. However, there is much evidence in this study to suggest that such differences are not related to infectious titre, but are indeed a true reflection of strain-specific properties. The detection of PrP^{res} in different CJD tissues is proposed to

correspond to the presence of infectivity. Western blot analysis of the different sCJD brain inocula showed that PrP^{res} was readily detected in all brain extracts and that the intensity of the PrP^{res} signal was comparable between the different inocula (Chapter 3). If PrP^{res} correlates with infectivity, this would suggest that infectivity levels were broadly similar in the different sCJD brain inocula. Furthermore, in the challenges from other CJD tissue types, the successful transmission of infectivity occurred from vCJD tonsil, appendix and TRG tissue, tissues in which levels of PrP^{res} were lower than could be detected by conventional Western blot analysis. Although vCJD is a different agent strain from sCJD and transmits more readily in wild-type mice, this may be supporting evidence evidence that titres of infectivity are likely not to be responsible for the differences in the transmission efficiency.

7.2.1.1 Comparison of transmission properties between wild-type mice and human transgenic mice after experimental transmission with brain homogenate from sporadic CJD subgroups

To further understand the role that host *PRNP* codon 129 genotype has in defining transmission and propagation of sCJD strains, three transgenic mouse lines were produced by gene targeting with the direct replacement of the mouse prion protein open reading frame with the three different human *PRNP* sequences (129MM, MV, VV). These mice designated HuMM, HuMV and HuVV were inoculated with brain homogenate from each sCJD subgroup in a directly comparable study to that undertaken in this thesis (Bishop, 2008; Bishop et al., 2010). In fact, five of the six sCJD cases selected for experimental challenge to the human transgenic mice were those that had previously been used for the inoculation of wild-type mice reported in this thesis. Therefore in the discussion of some of the data generated in this study, it is useful to draw a comparison with data from the human transgenic mice.

Similarities and differences emerge when comparing the two studies. By employing the identical strain typing parameters to those used in this thesis, inoculations of the transgenic mice demonstrated that the six sCJD subgroups behave as four distinct strains of agent (MM1 and MV1; MM2; MV2 and VV2; VV1) (Bishop, 2008; Bishop et al., 2010). These identical groupings have also been reported using two

different *in vitro* assays, one examining the protease sensitivity and conformational stability of PrP^{Sc} in different sCJD subgroups (Uro-Coste et al., 2008) and a second using the recently described PMCA method to investigate the efficiency of the different sCJD subgroups in the conversion of PrP^C to PrP^{Sc} (Jones et al., 2008). The grouping of these sCJD subgroups as distinct strains is closely similar to that reported in this thesis, supporting evidence that wild-type mice can be used to distinguish some sCJD strains. Where differences occur between the challenge of wild-type mice and the transgenic mice described by Bishop and co-workers, the differences may be explained by the resistance of the wild-type mice to infection with certain forms of sCJD agent. As a consequence, wild-type mice are not able to further distinguish between sCJD MV2, VV2 and VV1 inocula. In the transgenic mouse study, Bishop and co-workers showed that the HuMM and HuMV mice had similar levels of clinical disease and mean incubation periods for four of the six sCJD brain inocula (MM1, MV1, MV2 and VV2). These levels of clinical disease and incubation periods differed from those found in the HuVV mice challenged with the same four brain inocula. Few animals showed clinical disease following inoculation with the VV1 subtype in any of the three transgenic lines (Bishop, 2008; Bishop et al., 2010). This implies that transmission properties in the mice are influenced by the presence of methionine at codon 129 in the host. Therefore, the strain-related properties observed in the sCJD inoculated mice in this thesis may in part be a reflection the mice having the methionine allele at the corresponding mouse *Prn-p* codon 128.

However, data generated from the experimental challenge of sCJD MV2 inocula to wild-type mice in this thesis and in the experimental challenge of sCJD MM2 inocula in the corresponding human transgenic mouse model (Bishop, 2008; Bishop et al., 2010) have demonstrated that sCJD transmission properties are not solely defined by the codon 129 genotype of the donor and host. A complex relationship with the PrP^{res} type of the sCJD donor has also been demonstrated. In general, the presence of type 1 PrP^{res} in sCJD MM and MV donors appears to facilitate transmission across all four mouse lines examined in this thesis. With the exception of sCJD MM2 in RIII mice, cases with type 2 PrP^{res} largely failed to transmit disease in the wild-mice.

Similarly, all three transgenic lines (HuMM, HuMV and HuVV) were readily susceptible to disease with the appearance of clinical signs following inoculation of a sCJD MM1 brain inoculum, whereas no evidence of a TSE was observed in any mouse following challenge with a sCJD MM2 brain inoculum (Bishop, 2008; Bishop et al., 2010). Thus in terms of risk assessment, both studies suggest that the MM1 and MV1 subtypes, the most common “classical” form of sCJD, represent the greatest risk of secondary transmission of sCJD. Data from this thesis could also be used to suggest that the presence of type 2 PrP^{res} in the brain of sCJD cases may offer reduced risk of secondary human transmission of sCJD. However, when the full data set including the human transgenic mice is considered, this is not the case. The HuVV mice challenged with type 2 PrP^{res} from sCJD MV and VV donors produce the shortest incubation periods observed in any of the sCJD inoculations. In fact, data from the transgenic study suggest that VV individuals may be the most susceptible to secondary sCJD transmission with the observation that the HuVV mice developed clinical disease after challenge with five of the six brain inocula. Therefore, the risk of transmission of sCJD may be a function of the nature and source of the infectivity and the *PRNP* codon 129 genotype of the exposed individuals.

The relationship between transmissibility, host *PRNP* codon 129 genotype and the inoculation of different sCJD strains may already have been inadvertently demonstrated in humans. In a study investigating the distribution of *PRNP* codon 129 in human growth hormone (hGH) transmitted CJD patients in France and UK, different genotypic distributions were observed between the two countries (Brandel et al., 2003). The French hGH related patients showed a bias towards the MM genotypes whereas UK patients showed a bias towards MV and VV genotypes. Mechanisms of production of the hGH, the number of hGH treatments received and the duration in treatment was similar in patients from both countries; however, the actual growth hormone was prepared independently in France and the UK. One of the possibilities raised in this study was that different contaminating sCJD subtypes were used in the preparation of hGH in France and the UK. If, as data in this thesis supports, the different sCJD subtypes reflect different sCJD strains, the distribution of *PRNP* codon 129 genotypes in the French and UK cases might be due to infection

with different contaminating CJD strains. If the results of the human transgenic mice are a true reflection of what occurs in the secondary transmission of sCJD, this would suggest that the French patients were more than likely infected with hGH contaminated from sCJD MM1 or MV1 patients whereas the UK patients were more than likely contaminated with hGH from either sCJD MV2 or VV2 patients.

Data from this thesis and that described by Bishop and co-workers also support findings from a large-scale biochemical study investigating PrP^{res} types in 170 cases of sCJD (Head et al., 2004a). This study suggested that sCJD patients who have methionine homozygosity at *PRNP* codon 129 may be more predisposed to propagate type 1 PrP^{res} in the brain, whereas patients who are valine homozygous are more predisposed to propagate type 2 PrP^{res}. In this thesis, the propagation of type 1 PrP^{res} in a single RIII mouse following inoculation with brain homogenate from a sCJD MV2 patient is proposed to have resulted from the presence of a minority type 1 component in the inoculum (Chapter 4). However, the possibility that this may be a result of a predisposition of the mice to propagate type 1 PrP^{res} may be an alternative explanation. Results from the human transgenic mice provide supporting evidence for this proposition; the HuMM and HuMV mice also propagated type 1 PrP^{res} following challenge with a sCJD MV2 inoculum, whereas a PrP^{res} with a mobility associated with PrP^{res} type 2 was detected in the HuVV mouse line following inoculation with the same brain homogenate. Nevertheless, the observation of PrP^{res} type 2A in the mice in this thesis following challenge with brain homogenate from a sCJD MM2 case indicates that although there may be a bias to the propagation of type 1 PrP^{res}, the propagation of a type 2 PrP^{res} can be supported in the presence of the methionine allele at *PRNP* codon 129 in the donor tissue and host. This has also been supported by the successful transmission of vCJD in mice (Chapter 5).

It is interesting that the one sCJD MM2 brain homogenate inoculated in the transgenic mouse lines showed no evidence of transmission (Bishop, 2008; Bishop et al., 2010). As only a single MM2 case was used to challenge the transgenic mice the data must be interpreted with caution. However, Bishop and co-workers provide no alternative explanation of whether this lack of transmission may be attributed to a

resistance of the mice to infection from this sCJD subgroup or whether it may be a reflection of lower titres of infectivity in this inoculum. As brain homogenate from this same sCJD case produced evidence of a TSE in the wild-type mice in this thesis and in an earlier thesis using a different human transgenic mouse model (Wiseman, 2006), and that in all three studies inoculum was prepared from the same brain region, it is therefore unlikely that low titres alone are the reason. It is noteworthy that the transmission of brain homogenate from this sCJD MM2 patient transmitted more readily in the wild-type mice than the transgenic mice, even in the HuMM mouse line which had *PRNP* codon 129 genotype matching the donor. This is similar to that found with vCJD.

Conservation of PrP^{res} type following transmission is an integral part of the proposal that differences in the biochemical properties of PrP (conformation and glycosylation states) may play a role in enciphering strain-specific differences. In general, PrP^{res} typing data generated in this study supports this proposal. However, when combining the wild-type mouse data in this study with that generated from the human transgenic models described by Bishop and co-workers, in which alterations in the PrP^{res} type are observed following transmission, a more likely explanation is that PrP^{res} types are not a specific marker for agent strain, but are a result of the interaction between the strain and a host.

7.2.2 The co-occurrence of PrP^{res} types in sporadic CJD: implications for disease phenotype and agent strain

At the outset of this thesis the question of distinct TSE strains in sCJD focused on the six clinico-pathological subtypes of sCJD described by Parchi and Gambetti (MM1 / MV1, VV1, MM2cortical, MM2thalamic, MV2 and VV2) (Parchi et al., 1999) and the proposal that strain-specific information in sCJD is encoded by which of the two PrP^{res} types (type 1 or type 2A) is observed in individual cases combined with their *PRNP* codon 129 genotype. However, as this study has progressed, questions about the validity of this molecular-based sub-classification system and the relationship between disease phenotype, PrP^{res} type and agent strain have arisen with reports that a much larger proportion of sCJD cases than initially described contain both type 1

and type 2 (Puoti et al., 1999; Polymenidou et al., 2005; Yull et al., 2006; Schoch et al., 2006; Uro-Coste et al., 2008). In response to these reports, two recent papers have re-addressed the sub-classification of sCJD proposing that mixed PrP^{res} types in the brain are associated with further defined subtypes of sCJD (Parchi et al., 2009; Cali et al., 2009). Both reports propose that if thorough clinical investigations and extensive sampling of different brain regions, and the use of antibodies that have different affinities for types 1 and 2 are employed, then sCJD cases with mixed PrP^{res} types can be distinguished from ‘pure type 1’ and ‘pure type 2’ sCJD cases as distinct clinico-pathological phenotypes.

So what does this mean for the molecular definition of agent strain? Certainly, the data from this thesis and that of previous transmission studies in different animal models (Nonno et al., 2006; Bishop, 2008; Bishop et al., 2010) support the relationship between sCJD phenotypic variability and the propagation of unique strains. How these mixed PrP^{res} types will propagate in the mice is uncertain. However, transmission of brain tissue from the probable case of mixed PrP^{res} types carried out as part of this thesis does provide some preliminary evidence that is discussed later in this section. Will transmission of such cases produce a unique phenotype in the mice distinct from that observed from transmission of ‘pure type 1’ and ‘pure type 2’ cases, indicating the propagation of further distinct sCJD strains? As the sCJD phenotypes of these mixed PrP^{res} types are characterised by a co-existence of phenotypic features observed in ‘pure type 1’ and ‘pure type 2’ cases, it may be more likely that transmission studies may provide evidence of a co-infection of different TSE strains. In either case, transmission studies from mixed PrP^{res} type brain homogenate will play a significant role in answering these questions and the sCJD transmissions carried out in this study will provide a valuable standard on which to compare data from such studies.

Perhaps the most valuable information would come from the transmission of sCJD MM 1+2 cases in the wild-type panel and in the human transgenic lines used by Bishop and co-workers. Pure PrP^{res} type 1 and type 2 cases in this genotype had shown the most observable difference in the clinico-pathological and biochemical

phenotype after transmission in both mouse models. If transmission of such cases results in co-infection with distinct TSE strains, it would be predicted that a proportion of wild-type mice challenged would demonstrate a type 1 disease phenotype and a proportion show type 2 phenotype. Alternatively, it may be that a single disease phenotype will be selected and amplified by the host, depending on the host *PRNP* codon 129 genotype. This may be an alternative explanation for the apparent selection of a type 1 disease phenotype in the wild-type mice following transmission with the probable mixed sCJD MM1 +2 case (CJD 11) investigated in this study. Such selection of a single disease phenotype following challenge with mixed sCJD PrP^{res} types was demonstrated in a recent study investigating the clinico-pathological and biochemical characteristics of the sCJD agent following transmission in the squirrel monkey (Williams et al., 2007). In this study, brain homogenate from a single sCJD MM1 and MV2 patient were pooled prior to inoculation. Biochemical typing of PrP^{res} in frozen tissue samples from the frontal cortex of the sCJD inoculated squirrel monkeys detected only a single PrP^{res} type with a glycoform ratio and mobility resembling type 1 PrP^{res}. There are two possible explanations for these results. Firstly, squirrel monkeys, like wild-type mice, are uniformly homozygous for methionine at codon 129. Therefore, as suggested in this and in previous transmission studies, it may be that the MM1 component will transmit more successfully in MM hosts and will be replicate preferentially over the other sCJD MV2 component. This first alternative could be characterised as a competition/selection model. Alternatively, these results may provide further evidence that hosts who are methionine homozygous at codon 129 may be more predisposed to propagate type 1 PrP^{res} in the brain, irrespective of the nature of the material used in the challenge. This second alternative has more in common with a mutation model in which there is a switch from one conformation to another due to a 'transmission barrier'. The current uncertainties surrounding mixed PrP^{res} types raise the question of whether the existence of different sCJD strains can be properly investigated whilst the phenotypic variability of the disease has yet to be established and a full an accurate description of the contributions of different PrP^{res} types is made.

7.2.3 Transmissions of variant CJD demonstrate uniform and conserved BSE-related phenotypic properties on primary passage in wild-type mice

The remarkable uniformity observed in the clinical presentation and pathological features of vCJD patients, combined with the observation of a single PrP^{res} type associated with vCJD cases lead to the proposal that this human TSE is caused by infection with a single strain of agent. Data from this thesis provides strong support for this hypothesis as illustrated by the remarkable similarity in the transmission properties in the wild-type panel following primary challenge with brain homogenate from 10 cases of vCJD. The findings from the vCJD transmissions carried out in this thesis were published in 2009 in the Journal of General Virology (JGV) (see appendix) and provide a full characterisation of the vCJD agent strain after primary transmission and on subsequent passage in mice, thus providing a baseline on which agent strain in any future cases of suspected vCJD in other *PRNP* codon 129 subgroups (MV and VV) can be compared.

Early epidemiological (Will et al., 1996), biochemical PrP^{res} typing (Collinge et al., 1996) and experimental transmission studies (Bruce et al., 1997) all supported the proposal that the single strain of agent responsible for BSE is also responsible for vCJD. Data in this thesis provides further strong supportive evidence for this proposal. Incubation periods from the 10 vCJD challenges in this thesis are consistent with published data of BSE transmissions in the same mouse lines (Bruce et al., 1994; Bruce et al., 1997; Ritchie et al., 2009). Furthermore, the incubation period difference of around 100 days which is observed between the RIII and C57BL mice (both *Prn-p^a* mice), which is characteristic of the BSE agent (Bruce et al., 1994; Ritchie et al., 2009), was replicated in all seven transmissions from vCJD cases in which both mouse lines were included. In addition, sub-passage of vCJD brain isolates in different mouse *Prn-p* genotypes resulted in the isolation of two distinct mouse-passaged strains, closely similar to the 301V and 301C strains which were derived by serial passage of BSE in VM and C57BL mice respectively (Bruce et al., 2002; Ritchie et al., 2009). The biochemical phenotype of the vCJD inoculated mice in this study also mirrored that of BSE in cattle and the BSE agent in the same mouse lines, characterised by the predominance in the diglycosylated form of the protein.

In addition, the appearance of the unglycosylated fragment of the protein as a doublet in the vCJD inoculated mice after primary and on subsequent serial mouse passage also resembles a phenomenon previously reported to occur in BSE-derived 301V and 301C strains (Somerville et al., 2005), thus providing a further biochemical characteristic shared by BSE and vCJD.

The isolation of distinct mouse-passaged strains of agent on serial passage in different mouse *Prn-p* genotypes is not a unique feature of vCJD and BSE and has been reported previously in the mouse passage of different scrapie strains (Bruce et al., 2002). Interestingly in this thesis, sub-passage of sCJD MM1 brain isolates also resulted in two distinct mouse-passaged strains. Although different from the vCJD/BSE-passaged strains, the two sCJD-passaged strains were also isolated according to incubation period differences observed in the different mouse *Prn-p* genotypes. This may be indicative that the isolation of these two distinct disease phenotypes on serial passage of sCJD and vCJD may be consistent with host-permitted selection of strains rather than any strain-specific differences in the TSE agent (Bruce, 2003).

7.2.4 The vCJD/BSE agent is distinct from those of sCJD

In the original vCJD strain typing paper, Bruce and co-workers showed that vCJD could be distinguished from the MM1 subgroup of sCJD by differences in the appearance of clinical signs and lesion profiles after inoculation of brain homogenate in RIII mice (Bruce et al., 1997). Furthermore, studies comparing the biochemical phenotype of vCJD with that of different sCJD subtypes following experimental challenge in humanised transgenic mice, also differentiated these two human TSEs (Collinge et al., 1996). These two studies provided early evidence that vCJD represented a new subtype of human TSE, distinct from sCJD. In this thesis, comparison of the transmission properties of mice following inoculation with brain homogenate from 10 vCJD patients (Chapter 5) with that of 27 sCJD patients from all six possible sCJD subgroups (Chapter 4), confirms these findings and extends the evidence that the vCJD strain is distinct from sCJD strains isolated in the wild-type panel. Overall, vCJD transmits disease efficiently in the wild-type mouse panel,

resulting in a progressive clinical disease with vacuolar degeneration in almost 100% of inoculated mice. Mice challenged with vCJD show a consistent heavy and widespread deposition of PrP in the brain following immunohistochemical analysis and demonstrate a unique PrP^{res} type after Western blot analysis. In contrast, sCJD subgroups transmit less efficiently in the wild-type panel. The development of clinical disease was generally restricted to RIII mice challenged with one sCJD MM2 brain homogenate with a mean incubation period of around 730 days, over 300 days longer than that of vCJD in the same mouse line. Targeting of vacuolar pathology in sCJD inoculated mice also differed to those observed in vCJD challenges. The similarity found in the lesion profiles between the different mouse *Prn-p* genotypes challenged with sCJD subgroups (MM1, MV1 and MM2 only) was in contrast to vCJD, in which the different mouse *Prn-p* genotypes were associated with different patterns of vacuolar pathology.

In addition to clinical features, one of the most dramatic differences between the vCJD and sCJD inoculated mice was in the immunohistochemical analysis of the prion protein in which sCJD inoculated mice showed only small focal granular deposits of PrP in the brain compared to the intense and widespread PrP deposits observed in vCJD mice. What are the possible reasons for this discordance? One possibility is that the low levels of PrP in the sCJD challenged mice are a result of the mice having been sacrificed prior to the onset of clinical signs. However, this is unlikely as immunohistochemical analysis of the small numbers of sCJD challenged mice sacrificed with clinical TSE disease showed no obvious increase in the intensity or distribution of PrP in the mouse brain when compared to sCJD challenged mice sacrificed for reasons other than clinical TSE. Furthermore, Western blot analysis confirmed the presence of PrP^{res} in CNS tissue from sCJD inoculated mice showing vacuolar pathology, with no observable differences in the intensity of the PrP^{res} signal to that observed in mice after primary challenge with vCJD. Therefore, it may be that the differences observed in the PrP deposition between these human TSEs in mice may relate to the methodologies employed. Western blot analysis has confirmed that the PrP^{res} type differs between the sCJD and vCJD inoculated mice and that there are both conformational and glycosylation differences in the prion

protein in these two diseases. Such biochemical differences in PrP may result in sufficient alterations in the structure and folding of the prion protein such that the antigen retrieval methods used in the immunohistochemical analysis of PrP were sufficient to unmask the antigenic sites on mouse PrP in the vCJD inoculated mice but were unable to fully uncover those on PrP in the sCJD inoculated mice. In this study, immunohistochemical analysis was carried out with a single antibody raised against the prion protein (6H4). It might be useful to further investigate the deposition of the prion protein using a range of antibodies raised against different epitopes on the prion protein to fully evaluate the deposition and distribution of PrP in the sCJD inoculated mouse brain. However, preliminary investigations on a range of anti-PrP antibodies have provided no changes in the pattern of PrP deposition in sCJD inoculated mice. The demonstration that PrP staining patterns can vary depending on the primary antibody used in the analysis was reported in a study investigating PrP deposition in human TSEs, comparing 10 monoclonal antibodies raised against different epitopes on the prion protein. This study reported that a course and a plaque-like deposit successfully labelled with all 10 antibodies whereas, labelling of granular and synaptic deposits showed greater variation between the different antibodies (Kovacs et al., 2002a). In man, vCJD is associated with an intense peri-cellular PrP deposition with the formation of amyloid plaques and plaque-like deposits (Ironside et al., 1996; Ironside et al., 2000). In comparison to vCJD, granular and synaptic deposits of PrP are characteristic of the MM1 and MV1 subtypes of sCJD (Parchi et al., 1999); these two sCJD subtypes showed the most efficient transmission in the mice used in this thesis. Therefore the different staining patterns observed between the sCJD and vCJD challenged mice may be a reflection of the differences in the aggregation state of PrP^{Sc} in the human donor tissue. Nevertheless, the immunohistochemical detection method used in this study was efficient in differentiating between vCJD and sCJD challenged mice. In conclusion, all transmission properties investigated in this thesis were able to differentiate between sCJD and vCJD inoculum, confirming that these two human TSEs represent distinct strains of agent.

7.2.5 No evidence of BSE-related strain properties in sCJD occurring in two dairy farmers

It is noteworthy, that the original vCJD strain typing experiment described by Bruce and co-workers was carried out at a period of time in which the relationship between BSE, vCJD and sCJD was not clear. This is also the case for some of the earliest experimental challenges carried out as part of this thesis. Therefore, in addition to characterising the agent strain in vCJD and comparing with BSE, the original article also investigated whether there was any relationship between cases of sCJD and exposure to BSE. The case cohort described in Bruce *et al.* (1997) included two cases of CJD in dairy farmers who, having had BSE in their herds, had potentially been exposed to the BSE agent either from BSE-infected cattle or from contaminated animal feed (These two CJD cases are identified as CJD 2 and CJD 4 in this thesis). Both farmers were homozygous for methionine at codon 129 on the *PRNP* gene. The question raised was whether these cases of CJD showed any evidence of a BSE-related strain. In the article, comparison of the clinical data and vacuolar pathology in RIII mice challenged with brain homogenate from these two sCJD cases showed no evidence of a BSE-related phenotype when compared to the same mouse line inoculated with vCJD and BSE. Furthermore, the clinical and pathological features were consistent with transmissions carried out with brain tissue from four further cases of sCJD in methionine homozygous individuals; two which had occurred after the BSE outbreak (identified in this thesis as CJD 1 and CJD 3) and two ‘historical’ cases in which the patients had died prior to the onset of BSE (identified in this thesis as CJD 5 and CJD 6). Data in this thesis confirms the initial findings and extends the analysis of these two incidences of sCJD in dairy farmers in a number of important aspects. Firstly, this thesis extended the analysis by increasing the number of transmitted cases of sCJD in methionine homozygous individuals to 12, providing complete analysis of clinical signs of disease and vacuolar pathology in a larger number of mouse lines. Secondly, this thesis included the biochemical analysis of PrP^{res} in the mice following challenge with brain homogenate from the farmers and finally, the transmission characteristics of one of the farmers (patient CJD 2) was analysed after a further two passages in mice. Results showed that patterns of vacuolar pathology in the two farmers was consistent with that of the further eight

sCJD MM1 cases examined in this thesis. Crucially, Western blot analysis of PrP^{res} in frozen mouse tissue from these two transmission experiments showed a glycosylation pattern and mobility in the unglycosylated form of the protein closely resembling that of type 1 sCJD. This PrP^{res} type was consistent with that detected in the inocula used in the experimental challenges and consistent with the PrP^{res} type observed in the mice after transmission of the other sCJD MM1 cases. In summary, the data presented in this study provides further evidence that the CJD diagnosed in these two dairy farmers was sporadic in nature and argues against a link between occupational exposure to the BSE agent and the development of CJD.

7.2.6 No evidence of more than one vCJD/BSE-related agent strain

The existence of more than one strain of cattle BSE has been proposed following the identification of two atypical molecular phenotypes (H and L-type) by Western blot analysis of cattle subjected to routine slaughterhouse testing (Biacabe et al., 2004; Casalone et al., 2004). Although defined primarily by differences in their biochemical properties, neuropathological analysis of the L-type of atypical BSE also showed some distinctions from classical BSE, with the observation of PrP-immunopositive amyloid plaques in the brain. This provided further support that these atypical cases could represent further BSE strains (Casalone et al., 2004). Findings from the experimental transmission of BSE and vCJD in different lines of humanised transgenic mice and wild-type mice have also provided evidence for the existence of more than one BSE strain (Asante et al., 2002; Lloyd et al., 2004a; Beringue et al., 2008).

In the first series of experiments investigating the BSE to human ‘species barrier’, two lines of humanised transgenic mice (Tg35 and Tg45) expressing human PrP and encoding methionine at codon 129, showed a complex pattern of transmission following challenge with BSE and vCJD isolates (Asante et al., 2002). In this study, vCJD transmission to both Tg35 and Tg45 mice produced a neuropathology and biochemical phenotype in the mice consistent with that of human vCJD. This vCJD-like profile was also seen in the Tg45 mice challenged with BSE. However, transmission of BSE to Tg35 mice resulted in two distinct phenotypes; one which

was indistinguishable from that of vCJD challenged Tg35 and Tg45 mice; and a second which showed a biochemical phenotype more commonly associated with the transmission of sCJD in the Tg35 mice (Asante et al., 2002). This data suggested the isolation of two distinct BSE strains in Tg45 mice. A criticism of this study was that the Tg35 and Tg45 mice were produced on a mixed genetic background. The biological parameters (incubation period and lesion profile) that distinguish between different TSE strains are known to be modified by different genetic backgrounds (Bruce, 1993; Moore et al., 1998). Therefore, it was proposed that the different transmission properties seen in the BSE-challenged Tg35 mice may be due to differences in the genetic background of individual mice. This proposal was supported by findings from the same study which showed that different lines of wild-type mice, challenge with either vCJD or BSE, produced two distinct biochemical profiles depending on the *Prn-p* genotype of the inoculated mice (Asante et al., 2002). In order to address this criticism, a subsequent study carried out a further characterisation of these mouse-adapted BSE strains on serial passage in a single wild-type mouse line (SJL) (Lloyd et al. 2004). Analysis of incubation period, pattern of PrP deposition and PrP^{res} type in the mice were consistent with the proposition that these mouse-adapted BSE strains represented the isolation of distinct BSE strains (Lloyd et al., 2004a).

Such divergence in TSE strains has also been demonstrated in vCJD. In a recent study, four vCJD isolates were transmitted to a different humanised transgenic mouse line (Tg650), also expressing human PrP encoding methionine at codon 129 (Beringue et al., 2008). Three of the brain isolates faithfully propagated a vCJD-like neuropathology and biochemical profile in the mice. However, Tg650 mice challenged with the fourth vCJD isolate propagated two distinct phenotypes; one exhibiting the vCJD-like profile seen with the other vCJD isolates; and a second with a neuropathology and PrP^{res} type which was more consistent with sCJD inoculated Tg650 mice (Beringue et al., 2008). Interestingly, analysis of the spleen from the Tg650 mice consistently demonstrated a vCJD-like PrP^{res} type, even in the mice with a sCJD-like PrP^{res} detected in the brain (Beringue et al., 2008).

The findings from these studies were interpreted by the authors as suggesting the existence of more than one strain of BSE. These studies also raised the possibility that some individuals infected with the BSE agent may present with a phenotype indistinguishable from that of sCJD; or that a variant strain of BSE agent is generated upon transmission from cattle to humans which could emerge following secondary human transmission of vCJD. This variant strain may also be indistinguishable from that of sCJD. Data from this thesis does not support the findings of the above, studies in a number of important aspects. Firstly in addressing the question of the existence of more than one BSE strain, this thesis has demonstrated that the transmission properties of all 10 vCJD isolates were highly reproducible in wild-type mice. This is consistent with the proposal that all 10 patients were infected with the same strain of agent. Secondly, in investigating the existence of novel BSE strains, this thesis has demonstrated that all mice analysed produced a neuropathology and PrP^{res} type consistent with that of cattle BSE in the same mouse lines (Bruce et al., 1994; Bruce et al., 1997; Somerville et al., 1997; Brown et al., 2003; Ritchie et al., 2009). Therefore this thesis provides no evidence for a secondary or minor novel BSE strain, or of the generation of variant strains following cattle-to-human transmission of the BSE agent. Thirdly, the study described by Asante and co-workers suggested that some humans infected with BSE may develop a clinical disease which is indistinguishable from that of sCJD (Asante et al., 2002). Again, data from the extensive series of transmissions analysed in this thesis shows no evidence to support this proposition. The dramatic differences observed in the efficiency in the transmission of brain homogenate from vCJD patients compared to that observed with sCJD isolates would facilitate in the identification of any BSE-like strain properties within sCJD challenged mice. The lack of any clinical disease in the sCJD inoculated mice; the low levels of PrP deposition in the brain following immunohistochemical analysis, and the absence of any vCJD/BSE biochemical phenotype in mice inoculated with brain homogenate from different sCJD subgroups, provide no evidence of an association between the sCJD cases investigated in this thesis and infection with the BSE agent. Lastly, the study by Beringue and co-workers proposes that a minor strain of BSE agent may be generated upon cattle-to-human transmission which may emerge on subsequent human-to-human

transmissions. In this thesis, the lack of any evidence of a sCJD-like disease phenotype in any of the mice inoculated with vCJD is further evidence against the proposals made in these previous studies.

More recently, a distinct biochemical phenotype has been observed following the experimental transmission of sCJD isolates in another humanised transgenic mouse model (Bishop, 2008; Bishop et al., 2010). In this mouse model, three transgenic mouse lines were produced using the gene targeting method by the direct replacement of the mouse prion protein open reading frame with the three different human *PRNP* sequence (129MM, MV and VV). Results from the inoculation of the different sCJD subgroups in these three transgenic mouse lines (HuMM, HuMV and HuVV) have been directly compared with the data presented in this study earlier in this Chapter (section 7.2.1.1). In these transgenic mouse models, inoculation of HuVV mice with a sCJD MV2 and a VV2 isolate, resulted in a PrP^{res} type showing a predominance in the diglycosylated fragment and a mobility in the unglycosylated fragment of around 19kDa, a Western blot profile more commonly associated with vCJD and BSE (Bishop, 2008; Bishop et al., 2010). However, the clinical and neuropathological features of these mice were distinct from that of vCJD previously described in these transgenic mouse lines (Bishop et al., 2006). No evidence of a vCJD/BSE-like biochemical profile was observed in any of the HuMM or HuMV challenged mice (Bishop, 2008; Bishop et al., 2010). The authors in this study were cautious not to draw any direct links between the detection of this PrP^{res} glycoform profile and the implication of a BSE-like strain in these sCJD isolates. Although PrP^{res} type is a valuable addition in the differentiation of the BSE strain, the same glycoform profile has been demonstrated in some experimental strains of scrapie (Somerville et al., 1997) and in FFI in humans (Telling et al., 1996). Furthermore, other changes in the PrP^{res} type on transmission were observed in the study by Bishop and co-workers, specifically, the demonstration of a type 1 PrP^{res} in the HuMM and HuMV lines following inoculation with brain homogenate from sCJD MV2 and VV2 subgroup. These findings suggest that the resulting PrP^{res} type in the inoculated mouse may be influenced by the host genotype of the mouse in combination with agent strain rather than enciphered by agent strain alone. These

transmission studies in transgenic mice further highlight the difficulties in fully understanding the relationship between PrP^{res} type and TSE strain. Although PrP^{res} isotyping may be a reliable diagnostic test, able to differentiate between vCJD and sCJD in people and may be a valuable addition in helping to define agent strain in experimental settings, such transgenic studies demonstrate the danger in over interpreting information on agent strain from the PrP^{res} type alone.

7.2.7 Experimental challenge of vCJD peripheral tissues shows no evidence of tissue-specific alterations in agent strain

The demonstration of a vCJD-like clinico-pathological and biochemical phenotype in mice following challenge with different peripheral tissue homogenates is further support that vCJD has resulted from infection with a single strain of agent. Two of the vCJD patients investigated had disease onsets in the year 2000, three years on from the most recent vCJD case selected in the transmission of brain tissue. This is a further indication that there has been no significant alteration in the agent strain as the disease progressed. Furthermore, the demonstration that the strain properties of the vCJD agent in different tissue types is similar to those demonstrated by brain isolates suggests that the strain properties of vCJD are not substantially altered by the tissue in which it replicates. These findings confirm that of an earlier study showing conservation of the vCJD biochemical profile in the spleen on transmission to humanised transgenic mice (Beringue et al., 2008). Therefore, the subtle differences that have been observed in the biochemical phenotype in the different vCJD tissue types (Head et al., 2004b) may not be an essential part in enciphering agent strain.

Data from this thesis indicates that strain typing in vCJD can be successfully carried out from tissues other than brain; however, the efficiency of the transmissions will vary between individual tissue types, presumably as a result of lower titres of infectivity in peripheral tissues. The ability to strain type from tissues other than brain, particularly from lymphoreticular tissues, may be valuable in future strain typing of cases of vCJD in other codon 129 *PRNP* genotype (MV and VV), should they occur. To date, evidence that these other *PRNP* genotypes are susceptible to infection with vCJD comes from asymptomatic individuals who had demonstrable

levels of PrP^{Sc} in lymphoreticular tissues only (Peden et al., 2004; Hilton et al., 2004b; Ironside et al., 2006; Peden et al., 2010). Therefore, strain typing in these cases will rely on transmission from lymphoreticular tissues rather than brain. These studies are in progress. As part of the continuing collaboration between the NCJDRSU and the NPU/NPD, transmission experiments are underway with brain and spleen tissue sampled from an asymptomatic case of vCJD blood-borne infection in an MV individual, previously reported by Peden *et al.*, (2004). This patient died of causes unrelated to a TSE but was known to have received a blood transfusion of non-leucodepleted red cells from a donor who subsequently went on to develop vCJD. Evidence of the abnormal prion protein in this patient was found in the spleen and a cervical lymph node but not in the brain. Inoculations of brain and spleen homogenate from this patient have been carried out in both humanised transgenic mouse lines (HuMM, HuMV and HuVV) and in a single wild-type line. Comparable inoculations are also underway with brain and spleen tissue from the vCJD blood donor to this patient. Preliminary data from these inoculations show clinical disease and vacuolar pathology, consistent with a TSE, in the transgenic and wild-type mice challenged with brain and spleen isolates from the vCJD donor. Furthermore, TSE disease has been confirmed in a proportion of the mice challenged with the spleen isolate from the asymptomatic MV blood recipient. No evidence of a TSE has been found in any mouse challenged with brain tissue from this same MV individual (unpublished data). Therefore, strain typing of the vCJD agent in the MV patient may rely solely on the transmission properties of the spleen homogenate. The characterisation of the vCJD agent in spleen tissue carried out in this thesis and published in the JGV (Ritchie et al., 2009), will be an important benchmark on which to compare the data from such future studies. The findings from this thesis and that of a previous study (Beringue et al., 2008) have shown that the strain properties of the agent are not substantially altered by the tissue in which it replicates. Therefore, any differences found in the transmission properties of the spleen isolates from the MV recipient and the vCJD blood donor will most likely be an indication that there are alterations in the agent strain associated with the different codon 129 genotype of the host, rather than associated with alterations associated with different tissue types.

7.2.8 The absence of any evidence of a TSE in mice challenged with non-CNS tissue does not rule out the presence of low levels of infectivity

Overall, data from the experimental transmission of different lymphoid tissues, neural tissues and biological fluids have confirmed that the presence of infectivity is concurrent with the demonstration of PrP^{Sc} in tissues, with infectivity detected to some degree in vCJD spleen, tonsil, lymph node, appendix, peripheral nerve, DRG and TRG. However, given the relative insensitivity of the mouse bioassay, the absence of any evidence of a TSE in the mice after challenge with vCJD CSF, bone marrow, buffy coat and plasma does not rule out the presence of low levels of infectivity in these tissues/fluids. This is best illustrated by the negative results from one vCJD lymph node (CJD 40), in which PrP^{Sc} had been confirmed by immunohistochemical analysis of this tissue at autopsy. Possible reasons for the failure of this tissue to transmit disease include the lower infectivity levels observed in peripheral tissues compare to that of vCJD brain, combined with the difficulties associated with the sampling of tissues showing a heterogeneous distribution of PrP^{Sc} as described in detail in Chapter 6.

Experimental transmission of infectivity in sheep by transfusion of blood sampled during the clinical and preclinical phase of sheep naturally infected with scrapie or experimentally infected by an oral dose of BSE, confirmed that infectivity can efficiently be transmitted via blood transfusion (Hunter et al., 2002). There is now convincing evidence to support the presence of infectivity in vCJD blood based on the five instances of vCJD infection (three clinical cases and two asymptomatic) following transfusion of blood and blood (Llewelyn et al., 2004; Peden et al., 2004; Wroe et al., 2006; Health Protection Agency, 2009b; Peden et al., 2010). However, the transmission of blood components (plasma and buffy coat) from four vCJD patients in this thesis failed to show any evidence of infection in the mice.

What could the reasons be for the lack of transmission in the mice? The most likely explanation is that the relative volumes of primary inoculum injected into the mice were sufficiently low to prevent disease in the mice. Titres of infectivity in vCJD blood are not known. However, the findings from a hamster scrapie (hamster scrapie

263K strain) model suggest that the measured titre of infected hamster blood is on average 10 infectious doses (ID) per ml during the preclinical phase rising to 100 ID per ml during the clinical phase (Holada et al., 2002; Gregori et al., 2004; Gregori et al., 2006). If similar levels of infectivity are associated with blood from vCJD patients, and if blood is homogeneous, then a volume of 10-100µl or more would be required for the transmission of disease, even if there was no 'species barrier'. In the inoculations carried out in this thesis, 20µl volumes of either buffy coat or plasma were inoculated intracerebrally. Depending on the exact levels of infectivity in human blood and the extent of the 'species barrier', then 20µl volumes may be at the border line for successful transmission and it might be predicted that the appearance of a TSE in the mice would appear a 'hit or a miss'. This also explains why the sheep model has proven to be the most successful model for the demonstration of infectivity in blood collected from sheep incubating scrapie and experimental BSE (Houston et al., 2000; Hunter et al., 2002; Houston et al., 2008). In these sheep studies, individual sheep were transfused with a minimum of 200ml of blood collected from TSE-infected sheep. If the ID present in blood collected from sheep infected with scrapie or experimental BSE is comparable to the measured titre of infected hamster blood (Holada et al., 2002; Gregori et al., 2004; Gregori et al., 2006) this suggests that sheep transfused with TSE-infected blood would have received 2000 – 20,000 ID. This also explains the evidence for the successful secondary human transmission of vCJD by blood transfusion. In humans, the typical volume of blood transfused is one unit (around 400mls). Again, if similar to that measured in infected hamster blood, this would be associated with 4,000 ID in a (preclinical) vCJD-infected blood donation. It is also possible that the route of infection may be important in the transmission of infection from blood. It would appear through the secondary human transmission of infectivity via blood transfusion that the transmission of infection from vCJD blood is highly efficient via the intravenous route. In the experiments described in this study, all inoculations from peripheral tissues and fluids were carried out with a single IC injection.

7.2.9 Detection of infectivity in sCJD blood and its implications

Demonstrable levels of PrP^{Sc} have been found in the pituitary gland (Peden et al., 2007), spleen and intramuscular nerve fibres in a proportion of sCJD patients (Glatzel et al., 2003; Peden et al., 2006). However, peripheral tissue involvement in sCJD is thought to be more limited than that of vCJD (Head et al., 2004b). In this thesis, the lack of any evidence of disease in mice following challenge with a sCJD spleen homogenate appears to support this position. Therefore, the detection of infectivity in a sCJD buffy coat sample was unexpected, particularly in view of the negative results obtained from the transmission of vCJD blood components. Data from this thesis is not the first evidence of infectivity in sCJD blood. Previous transmissions in different rodent models have also provided unconfirmed reports of the transmission of infectivity from buffy coat (Manuelidis et al., 1985) and from whole blood (Tateishi, 1985) taken at autopsy from sCJD patients. In this thesis, the evidence of infectivity in sCJD buffy coat is based on the neuropathological changes of a single VM mouse.

One question which arises is whether this is true result or merely a laboratory error involving misidentification of samples. A laboratory error is thought to be highly improbable due to the procedures applied during these transmissions and an audit trail of this result failed to identify evidence of a misidentification of samples of mice. Therefore, it is assumed that this is a true result, which is to say that the mouse acquired a TSE which is consistent with the inoculum used to challenge it. Furthermore, immunohistochemical analysis for the prion protein in this one mouse showed the specific targeting and intensity of PrP deposits consistent with that of mice inoculated with sCJD brain isolates. Although contamination or experimental misidentification can not be completely ruled out, this result provides direct evidence of low levels of infectivity in sCJD blood and evidence supporting a peripheral involvement in sCJD. However, with such an unexpected result, it will be important to carry out further work to confirm it. This may involve the inoculation of this sCJD blood sample to humanised transgenic mice or analysis of the buffy coat sample biochemically, should a more sensitive method be available. If infectivity is associated with sCJD blood, one question that arises is why there are so few cases of

infectivity in the mice? Again the explanation may be associated with low infectious titres present in the inoculum. If at worst, titres of infectivity present in sCJD blood are similar to that of patients with vCJD then the transmission of disease in inoculations of animals with materials containing less than 1 ID per volume may become increasingly stochastic. However, it would be wrong to dismiss occasional positive transmission in such cases.

What might the implications be for risk assessment associated with the use of blood and blood products from sCJD patients? There have been a number of reports of sCJD arising in individuals who have had transfusion with blood or blood products (Klein and Dumble, 1993; Creange et al., 1995; de Silva, 1996; Patry et al., 1998; Hewitt et al., 2006); however, unlike vCJD, there is as yet no causal link between the blood donation in these cases and CJD in the blood donors. Therefore, the proposed risk from the secondary transmission of sCJD by blood products has been assumed to be theoretical and perceived to be much lower than that of vCJD. However, the results of this thesis suggests otherwise. What are the possible reasons for the apparent reduced risk in the secondary transmission of infectivity from sCJD blood compared to vCJD? In contrast to vCJD where the median age at death is 29 years, the median onset for sCJD is 68 years. In general, blood donations are made by younger people; however, the blood transfusion service states that any healthy individual between the age of 17 and 65 can donate blood (<http://www.blood.co.uk/can-i-give-blood/who-can-give-blood/>). The maximum age at which an individual can donate blood is younger than the average onset of sCJD, therefore the risk from sCJD-infected blood entering the blood pool is thought to be sufficiently low. Furthermore, the incubation period in sCJD may be short compared to vCJD further reducing the risk of sCJD patients donating blood while incubation the disease. Therefore although this study provides evidence of infectivity within sCJD blood the risks overall may be much lower for reasons on disease pathophysiology and blood donation patterns.

7.3 Summary of thesis findings

Work presented in this extensive series of transmission experiments has provided a comprehensive and thorough characterisation of the sCJD and vCJD agent on transmission to wild-type mice. The overall objective of this thesis was to investigate the relationship between disease phenotype, *PRNP* codon 129 genotype, PrP^{res} type and agent strain. In addition, this thesis also aimed to establish whether infectivity could be demonstrated in tissues other than brain in sCJD and vCJD and if so, whether there are any tissue-specific alterations in the agent strain in these tissue types. In addressing these aims, four specific questions were asked. These questions and the main findings from this thesis are summarised below.

1. Do the six different sCJD subgroups, as classified by *PRNP* codon 129 and PrP^{res} type behave as different strains of agent on transmission to wild-type mice?

Chapter 4 details the first direct comparison of all strain typing parameters in wild-type mice challenged with brain tissue from all six sCJD subgroups (as classified by *PRNP* codon 129 and PrP^{res} type). The findings have provided evidence that the six sCJD subgroups propagate as three distinct sCJD strains on transmission to wild-type mice (MM1/MV1; MM2; MV2/VV1/VV2). Further analyses of the groupings indicate that the transmission properties of sCJD in the mice are attributed to a combination of *PRNP* codon 129 genotype and PrP^{res} type of the donor. Tissue from patients with at least one methionine allele at codon 129 on the *PRNP* gene and who have a PrP^{res} type 1 were found to transmit most efficiently in this mouse model. Furthermore, the findings that PrP^{res} type is conserved on primary transmission and on subsequent serial mouse passage is consistent with the ‘prion hypothesis’ which proposes that distinct TSE strains are associated with differences in PrP^{Sc} conformation and glycosylation.

2. How do transmissions from sCJD, proposed to have more than one strain of agent compare with vCJD, believed to be a single strain of agent?

Data presented in Chapter 5 has provided a full characterisation of the vCJD agent in 10 vCJD brain isolates. Results were consistent with the proposal that all 10 patients were infected with the same strain of agent. Furthermore, transmission properties were consistent to those previously reported with BSE in the same mouse line, providing further evidence that BSE is the origin of these vCJD cases. Comparison of incubation period, lesion profile, patterns of PrP deposition and PrP^{res} type in the brain of vCJD and sCJD inoculated mice were consistent with the proposal that vCJD represents a distinct strain of agent to those propagated in sCJD transmissions.

3. Can the detection of infectivity in vCJD patients be extended to tissues other than brain, spleen and tonsil? Furthermore, can infectivity be detected outside the CNS in sCJD?

Experimental transmissions outlined in Chapter 6 have extended the evidence for a widespread lymphoid tissue infection and have provided the first direct demonstration of the presence of vCJD infectivity in the peripheral nervous system. Infectivity was detected in vCJD spleen, tonsil, lymph node, appendix, DRG, TRG, spinal cord and peripheral nerve. Consistent with the proposal that sCJD has a more limited peripheral involvement to that of vCJD; no evidence of transmission was found following challenge with one sCJD spleen isolate. However, the observation of infectivity consistent with a TSE following inoculation of a sCJD buffy coat indicates that low levels of infectivity may be present in blood from clinical cases of sCJD.

4. Is agent strain subject to any tissue specific modifications?

Chapter 6 details the incubation period, lesion profile and pattern of PrP^{res} deposition in mice challenged with different vCJD tissue types. The findings have shown a closely similar pattern to that found in vCJD brain transmissions (Chapter 5). This provides evidence that the vCJD agent is not substantially altered by the tissue in which it replicates.

7.4 Concluding remarks

This thesis has used “classical” strain typing in wild-type mice to characterise the agent strain in sCJD and vCJD. The majority of transmission work in TSEs now involves the use of humanised transgenic mouse models. Developed primarily to overcome the ‘species barrier’, transgenic models have also been used to investigate the effects of the polymorphism at codon 129 of the *PRNP* gene providing valuable information regarding the nature of TSE strains. Nevertheless, the findings from this thesis confirm that the traditional wild-type mouse panels developed at the NPU remain a highly successful and relevant tool in the investigation of human TSEs. This thesis demonstrates that wild-type mice are very efficient hosts for vCJD. This is in contrast to transgenic mice expressing human PrP which have been found to have a pronounced barrier to challenge with vCJD prions and are apparently resistant to infection with the BSE agent. Furthermore, the propagation of three sCJD strains in this thesis show close similarities to sCJD strains propagated in different humanised transgenic lines.

However, the findings from this thesis raise a number of important issues regarding the continued use of wild-type mice in the investigation of human TSEs. Firstly, are wild-type mice a valid tool in the investigation of sCJD if not all cases transmit and very few animals develop clinical signs? Secondly, this thesis has highlighted that the role of codon 129 in *PRNP* is paramount in influencing transmission. The difficulties observed in the transmission of some sCJD subgroups (MV1 and VV) to wild-type mice have been overcome using humanised transgenic mouse lines expressing the *PRNP* gene with the codon 129 MM, MV and VV genotypes. This questions whether the problems associated with “classical” strain typing could be overcome using gene targeting to produce mice with valine at position 128 in the murine *Prn-p* gene? Finally, can strain typing in wild-type mice be justified in view of the long incubation times, costs and the ethical considerations associated with the numbers of animals sacrificed, particularly in view of the lack of success in the transmission of some sCJD subgroups?

Recently the development of *in vitro* assays such as CDI to probe abnormal prion protein conformation, and models such as PMCA and cell culture systems to examine replication in a cell-free and cellular systems, have been proposed as alternatives to the use of animals in TSE research models (reviewed in Ryou and Mays, 2008; Jones et al., 2010). Similarly to transgenic mouse models, *in vitro* assays are proving an important tool in the better understanding of the species barrier effect, the effects of the *PRNP* codon 129 genotype and more recently in the propagation of TSE strains. There are clear advantages to the development and application of *in vitro* assays over animal models in TSE research. Primarily they offer a faster and cheaper model for the conversion of PrP^C or amplification of PrP^{Sc}. In addition, *in vitro* assays offer a more controllable environment in which to investigate TSEs. The ability to manipulate these *in vitro* are being utilised in the potential development of a sensitive diagnostic technique for TSE infectivity in, for example blood. Despite these technologies emerging as powerful tools they remain relatively poorly validated and incompletely understood. In the case of cell culture assays, there are currently no human cells that are able to stably replicate human TSE strains. In addition, there are still questions surrounding several reports of spontaneous PrP^{Sc} generation using the PMCA PrP^{Sc} amplification technology. Fundamentally, these *in vitro* cell-free conversion assays investigate TSEs at a molecular level focussing almost exclusively on PrP^{Sc} conversion. Unlike animal models, *in vitro* assays are not able to replicate TSE disease as such nor are they able to study the pathogenesis of TSEs at the cellular and tissue level. Animal models are able to investigate important specific pathological features of TSEs, such as lymphoreticular involvement, neuroinvasion and the targeting of pathology to particular regions and cell types in the brain. Therefore, at present this thesis contends that there remains a key role for the use of wild-type mice in the investigation of human TSEs.

Appendix

Data generated from this thesis was published in one article. The reprint of this article is presented in this appendix.

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Transmissions of variant Creutzfeldt–Jakob disease from brain and lymphoreticular tissue show uniform and conserved bovine spongiform encephalopathy-related phenotypic properties on primary and secondary passage in wild-type mice

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Prion strains are defined by their biological properties after transmission to wild-type mice, specifically by their incubation periods and patterns of vacuolar pathology ('lesion profiles'). Preliminary results from transmissions of variant Creutzfeldt–Jakob disease (vCJD) to wild-type mice provided the first compelling evidence for the close similarity of the vCJD agent to the agent causing bovine spongiform encephalopathy (BSE). Complete results from this investigation, including the transmission characteristics of vCJD from brain and peripheral tissues of 10 cases (after primary transmission and subsequent mouse-to-mouse passage), have now been analysed. All 10 vCJD sources resulted in consistent incubation periods and lesion profiles, suggesting that all 10 patients were infected with the same strain of agent. Incubation periods suggested that infectious titres may be subject to regional variation within the brain. Comparison of incubation periods and lesion profiles from transmission of brain and peripheral tissues showed no evidence of tissue-specific modification in the biological properties of the agent. Analysis of the protease-resistant prion protein (PrP^{res}) by Western blotting from primary and subsequent passages in mice showed a glycosylation pattern closely resembling that of vCJD in humans, the so-called BSE 'glycoform signature'. Minor variations in PrP^{res} fragment size were evident between mouse strains carrying different alleles of the gene encoding PrP both in primary transmissions and on further passages of vCJD brain. Overall, the results closely resembled those of previously reported transmissions of BSE in the same mouse strains, consistent with BSE being the origin of all of these vCJD cases.

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INTRODUCTION

It is now over 20 years since bovine spongiform encephalopathy (BSE) was first reported in cattle in the UK (Wells *et al.*, 1987). BSE is a transmissible spongiform encephalopathy (TSE) or prion disease, a member of a unique group of fatal neurodegenerative disorders that occur in humans as well as in a number of animal species. In 1996, a new and distinct human prion disease was identified in the UK, known as variant Creutzfeldt–Jakob disease (vCJD) (Will *et al.*, 1996). Experimental transmission of vCJD and BSE in animal models provided early evidence supporting the initial hypothesis that vCJD

emerged as a result of human infection with the BSE agent, most probably through the consumption of contaminated meat products (Collinge *et al.*, 1996; Lasmezas *et al.*, 1996; Bruce *et al.*, 1997; Hill *et al.*, 1997; Scott *et al.*, 1999).

A key characteristic of prion disorders is the deposition in the brain of an abnormal, protease-resistant form of a normal host-encoded protein, the prion protein (PrP). This abnormal protein (PrP^{Sc}) has been proposed to be the major, if not sole, component of the infectious agent (Prusiner, 1982). The existence of individual prion strains continues to raise the question of how a single misfolded protein can encode all of the information required for the demonstrable phenotypic diversity of prion diseases (Farquhar *et al.*, 1998). Prion strains are defined by their

A supplementary figure and three supplementary tables are available with the online version of this paper.

biological properties after transmission to inbred lines of wild-type mice, specifically by the incubation period and the distribution of vacuolar pathology in the brain (referred to as the lesion profile), which together provide a 'signature' of the biological properties of the agent (Fraser & Dickinson, 1968; Dickinson & Meikle, 1971; Bruce *et al.*, 1991). It has been proposed that the strain-specific biological properties may be encoded in different conformations and glycosylation states of PrP^{Sc} (Telling *et al.*, 1996). These different conformers and glycotypes are usually distinguished by the analysis of the protease-resistant core of PrP^{Sc} (referred to as PrP^{res}) after limited digestion with proteinase K and analysis by Western blotting; this is an approach commonly referred to as 'molecular strain typing' (Collinge *et al.*, 1996; Parchi *et al.*, 1996, 1999). A single unique PrP^{res} type is associated with vCJD cases, defined by a predominance of the diglycosylated form of PrP^{res} (Collinge *et al.*, 1996). This same unique biochemical glycotype is found with cases of natural BSE and other BSE-related conditions. This BSE 'glycoform signature' further supports the hypothesis that these diseases are linked by a common agent (Collinge *et al.*, 1996).

To date, all tested clinical cases of definite vCJD are homozygous for methionine at codon 129 on the human PrP gene (*PRNP*). The susceptibility of the other *PRNP* codon 129 subgroups to infection with the BSE agent and the development of clinical vCJD remain uncertain, although it appears that MV and VV individuals may also be susceptible to infection (Peden *et al.*, 2004; Hilton *et al.*, 2004b; Ironside *et al.*, 2006; Bishop *et al.*, 2006). In mice, three alleles of the mouse prion protein gene (*Prn-p*) have been described (Westaway *et al.*, 1987; Lloyd *et al.*, 2004). The vast majority of mouse strains analysed are either from the *Prn-p^a* (Leu-108, Thr-189) or *Prn-p^b* (Phe-108, Val-189) genotype (Westaway *et al.*, 1987). These two alleles exert a major influence on the incubation period of the disease, a major determinant of agent strain, with each prion strain having a distinct and highly reproducible pattern of incubation periods in the three possible *Prn-p* genotypes (the two homozygotes and the heterozygote F₁ cross) (Dickinson & Meikle, 1971; Bruce *et al.*, 1991). Different prion strains are often isolated from a single source by serial passage through mice of different *Prn-p* genotypes, so a full characterization of a source includes strain typing on both primary transmission to mice and subsequent mouse-to-mouse transmission of the isolate.

In vCJD, the detection of PrP^{Sc} in peripheral tissues has led to concerns over the possible secondary transmission of vCJD through blood transfusion and other iatrogenic routes. The existence of more than one BSE prion strain, which may have already infected humans, and which has a biochemical phenotype indistinguishable from that of sporadic CJD (sCJD), was suggested in a publication examining the transmission of BSE and vCJD in transgenic mice (Asante *et al.*, 2002). This study raised concerns that patients with a phenotype consistent with sCJD may

actually have acquired the disease from infection with the BSE agent (Asante *et al.*, 2002; Beringue *et al.*, 2008). These and other such concerns have prompted us to complete, collate and conduct a careful reappraisal of all the available data relating to transmission of vCJD to wild-type mice, including transmission from brain and peripheral tissues, pathological and biochemical assessment and analysis of selected subpassages.

METHODS

Creutzfeldt-Jakob disease (CJD) inocula. Frozen central nervous system (CNS) and peripheral tissue samples from 10 neuropathologically confirmed cases of vCJD were investigated. Consent and ethical approval for the retention and research use of tissues from these CJD cases had been obtained (LREC reference number LREC/2000/4/157). Grey-matter-enriched brain samples (approx. 2 g) from each vCJD case were taken from either the cerebellar hemisphere (*n*=3) or the frontal cortex (*n*=7). A 10% (w/v) brain homogenate was prepared in physiological saline as described previously (Bruce *et al.*, 1997). For transmission of vCJD from lymphoreticular tissues, samples (approx. 2 g) of spleen (*n*=1 vCJD case) and tonsil (*n*=2 vCJD cases) were prepared as brain samples. Subpassage of isolates derived from cerebellar samples of two different vCJD cases were carried out with a 1% (w/v) brain homogenate prepared from the brain of a single RIII, VM and C57BL mouse which showed unequivocal clinical and histological signs of a prion disease.

CJD transmissions. Three inbred mouse strains (RIII, VM and C57BL) and one cross (C57BL × VM) were challenged. RIII and C57BL strains are both of the *Prn-p^a* genotype, whereas VM is of the *Prn-p^b* genotype. In an initial study, anaesthetised mice of each strain were inoculated with brain homogenates by a combination of intracerebral (20 µl) and intraperitoneal (100 µl) routes. In a further study, RIII and VM mice were injected with brain, spleen or tonsil homogenates by the intracerebral route (20 µl) only. For subpassages, all three inbred mouse lines and the C57BL × VM cross were inoculated via a single intracerebral injection. Mice were scored weekly for signs of clinical neurological disease as described previously (Fraser & Dickinson, 1968). Brains were removed at a defined clinical end point with one lateral half of each mouse brain frozen at -20 °C for further passage in mice or for biochemical analysis; the other half of the brain was fixed in 10% formal saline for histology and PrP immunohistochemistry. Experimental transmissions from two cattle BSE sources (10% brain homogenates) were performed in the same mouse lines by a combination of the intracerebral and intraperitoneal routes as described for vCJD transmissions.

Incubation period and histological assessment. Incubation periods were calculated as the interval between injection and a defined clinical end-point, when mice showed unequivocal signs of neurological disease with confirmed positive vacuolar pathology (Dickinson & Meikle, 1971; Bruce *et al.*, 1997). Haematoxylin and eosin (H&E) staining was carried out on 5 µm paraffin sections from all mice. Vacuolar changes were scored in nine standard grey matter regions and three white matter regions using established criteria, and lesion profiles were constructed (Fraser & Dickinson, 1968).

PrP immunohistochemistry. Immunohistochemistry for PrP in 5 µm sections of formalin-fixed, paraffin-embedded mouse brain was carried out as described previously (Hilton *et al.*, 2004a). PrP was detected with the monoclonal antibody 6H4 (Prionics) and PrP was visualized using the CSA amplification system (Sabatini *et al.*, 1998).

Biochemical analysis of PrP^{res} by Western blotting. Western blot typing of PrP^{res} was carried out as described previously (Head *et al.*, 2004), except that the monoclonal antibody 6H4 was used.

RESULTS

All vCJD brain isolates transmitted successfully to all three mouse strains and to the C57BL × VM cross, with the appearance of clinical and/or pathological signs associated with prion disease (Supplementary Table S1, available in JGV Online).

Incubation periods and lesion profiles

All transmissions of vCJD CNS tissue showed the same order of appearance of clinical symptoms in the mouse strains (Fig. 1a). The shortest incubation periods were observed in RIII mice and the longest were found in the C57BL × VM cross. Incubation periods were broadly similar irrespective of the route of injection or CNS tissue (frontal cortex or cerebellum) used for inoculation. However, the exact timing of the appearance of clinical symptoms showed some variability between individual vCJD cases, most noticeably with inoculum prepared from the frontal cortex of the brain. Inoculum prepared from the cerebellar hemisphere consistently resulted in shorter incubation periods when compared with inoculum prepared from the frontal cortex, suggesting a higher titre of infectivity. Incubation periods closely resembled those

observed in transmission of BSE from two unrelated cattle, using the same protocol (Fig. 1b). In general, lesion profiling showed a similar pattern for each vCJD isolate regardless of brain region or route of inoculation (Fig. 2a). There was some variability in the intensity of vacuolation between individual vCJD sources, most noticeably in the C57BL × VM cross; however, the general pattern of vacuolation was similar. Lesion profiles did differ between mouse strains of different PrP genotypes, with the *Prn-p^a* genotype (RIII and C57BL mice) showing a pattern distinct from that of the *Prn-p^b* genotype (VM) and the *Prn-p^{ab}* cross (C57BL × VM). Lesion profiles resembled those of primary BSE transmissions (Fig. 2a).

Immunohistochemical localization of PrP in primary transmissions

Immunohistochemical detection of PrP showed a widespread accumulation of PrP^{Sc} throughout the brains of infected mice, most prominently in the medulla, thalamus and around areas of intense vacuolation (Fig. 3a, b). Subtle differences in the distribution of PrP within the hippocampus region were observed between the different *Prn-p* genotypes. Amyloid plaques, a common feature of some forms of CJD including vCJD, were not a prominent feature in the mice and were observed only in the VM and C57BL × VM cross, mainly restricted to the corpus callosum (Fig. 3b). Levels of PrP immunostaining appeared higher in the *Prn-p^b* mice and the C57BL × VM cross compared to *Prn-p^a* mice.

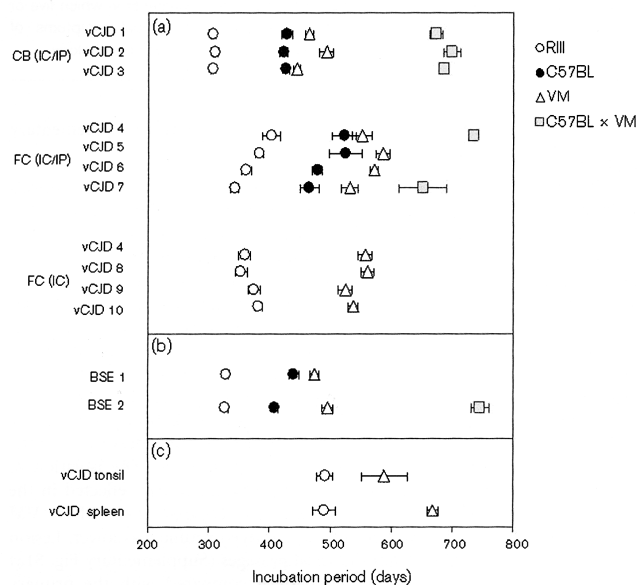


Fig. 1. Mean (\pm SEM) incubation periods in wild-type mice challenged with brain homogenate from vCJD sources (a), brain homogenate from two unrelated cases of cattle BSE (b) and vCJD tonsil and spleen tissue (c). Mean (\pm SEM) incubation periods for spleen are taken from vCJD 4, and for tonsil are taken from the combined incubation periods from vCJD 4 and vCJD 8. FC, Frontal cortex; CB, cerebellar cortex; IP, intraperitoneal injection; IC, intracerebral injection.

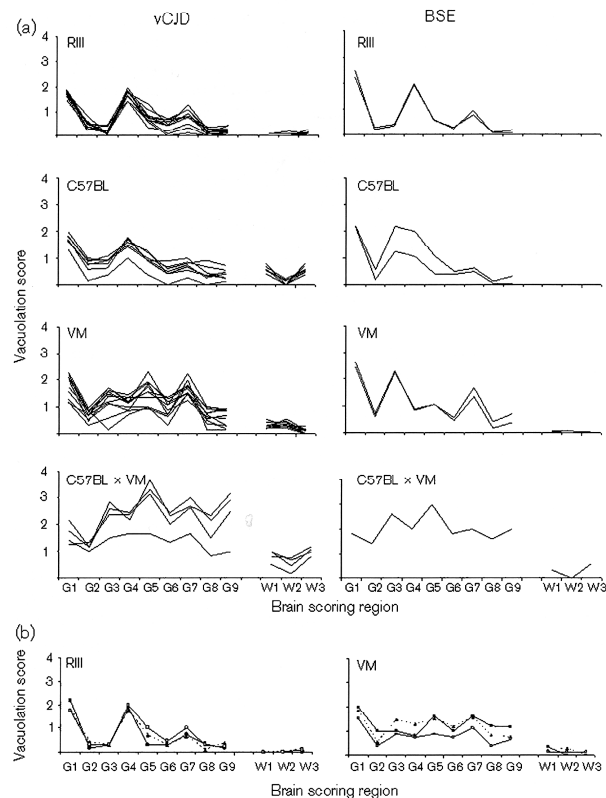


Fig. 2. Mean lesion profiles for transmissions from vCJD brain homogenate and cattle BSE into wild-type mice (a) and vCJD brain (▲, dotted line), spleen (▲, solid line) and tonsil (□) into VM and RIII mice (b) (lesion profiles for brain and tonsil are constructed from the combined vacuolation scores from vCJD 4 and vCJD 8). Lesion profiles were constructed from transmission experiments in which five or more mice produced clinical symptoms of disease with positive vacuolar pathology.

Biochemical analysis of PrP^{res}

Primary vCJD brain transmissions produced a glycosylation pattern closely resembling that of vCJD in human brain tissue, which was characterized by the predominance of the diglycosylated form of the protein (Fig. 4a). A feature of the experimentally infected mice that was not observed in vCJD in humans was the appearance of the unglycosylated fragment as a doublet, composed of distinct upper and lower bands. The relative amounts of these two bands varied and this correlated to a large extent with the mouse *Prn-p* genotype; the lower band predominated in *Prn-p^a* mice, whereas the upper band predominated in *Prn-p^b* mice. Both bands were readily discernible in the genetic cross (Fig. 4a).

Mouse-to-mouse passage

Both subpassages provided very similar results for all strain typing parameters examined, with the incidence of clinical and pathological features of disease being 100% in most

groups and close to 100% in the others (Supplementary Table S2).

On further mouse-to-mouse passage, incubation periods shortened dramatically, indicative of the removal of a species barrier. Subpassage in *Prn-p^a* mice (C57BL or RIII) resulted in the incubation periods in the mouse genotypes (Fig. 5b) being identical to those seen in primary transmissions (Fig. 5a), whereas subpassage in *Prn-p^b* mice (VM) changed that order, with very short incubation periods in *Prn-p^b* mice (Fig. 5c). These distinct incubation period rankings indicate that serial mouse passage resulted in the isolation of two distinct agent strains, determined by host genetics and recognized by incubation period. These results closely resembled those of serial passage of cattle BSE in the same mouse strains (Fig. 5). The isolation of two different agent strains was not clearly reflected in the lesion profile results, except in the case of C57BL × VM mice, in which the VM-passaged profile was lower. Lesion profiling from the serial passages (Supplementary Fig. S1a) showed a modified pattern compared with the primary

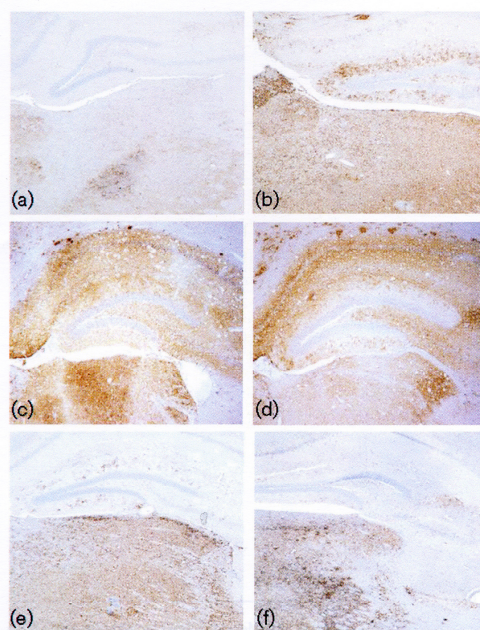


Fig. 3. (a, b) Immunohistochemistry for the prion protein in an RIII (a) and VM (b) mouse challenged with vCJD brain homogenate. (c, d) PrP accumulation within the hippocampus of an RIII mouse after first mouse-to-mouse passage in *Prn-p^a* (RIII) (c) and *Prn-p^b* (VM) (d) mice. (e, f) PrP labelling in the thalamus and hippocampus in a VM mouse challenged with vCJD spleen tissue (e) and an RIII mouse challenged with vCJD tonsil tissue (f).

transmissions (Fig. 2a), but a similar pattern to that found for mouse-passaged BSE (Supplementary Fig. S1b). No significant differences were found between first and second mouse passages. Slight but consistent differences did occur between the recipient mouse strains, again showing the influence of host genetic factors on lesion profiles (Supplementary Fig. S1a).

PrP deposition in the brain was more intense on serial passage compared with the primary transmissions (Fig. 3c, d). The general distribution of PrP was similar to that observed on primary transmission, with the subtle differences observed in the PrP deposition within the hippocampus between the different *Prn-p* genotypes conserved on serial passage. In contrast with the primary transmissions, all four mouse strains passaged in *Prn-p^a* and *Prn-p^b* mice showed the presence of amyloid plaques, mainly restricted to the corpus callosum. No significant differences in patterns of PrP accumulation were observed between first and second passage or between *Prn-p^a*- and

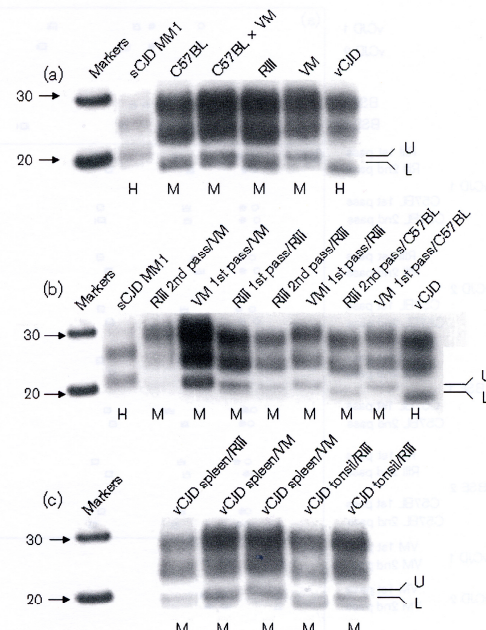


Fig. 4. Western blot analysis of CNS tissue from wild-type mice after experimental transmissions of vCJD brain tissue (a), subsequent first and second mouse-to-mouse passage (b) and vCJD tonsil and spleen tissue (c). Molecular weight markers are shown in kDa. H, human brain tissue; M, mouse brain; U, upper band of the unglycosylated fragment; L, lower band of the unglycosylated fragment.

Prn-p^b-passaged isolates (Fig. 3c, d). Western blot analysis of PrP^{res} in mice produced a glycosylation pattern similar to that of vCJD in human tissue and in vCJD primary mouse passages. No changes were observed between first and second passage, although variation in the relative abundance of the upper and lower unglycosylated doublet was seen. Unlike the primary transmission results, there was no simple direct correlation between *Prn-p* genotype and the composition of the doublet (Fig. 4b).

Transmission of lymphoreticular tissue

Clinical and pathological signs associated with prion disease were observed in RIII and VM mice challenged with vCJD spleen and tonsil tissue (Supplementary Table S3). Overall, transmission of vCJD from spleen and tonsil resulted in extended incubation periods compared with vCJD brain – this was most obvious with spleen tissue – indicating that infectious titres in these lymphoid tissues may be lower than those in the corresponding vCJD brain

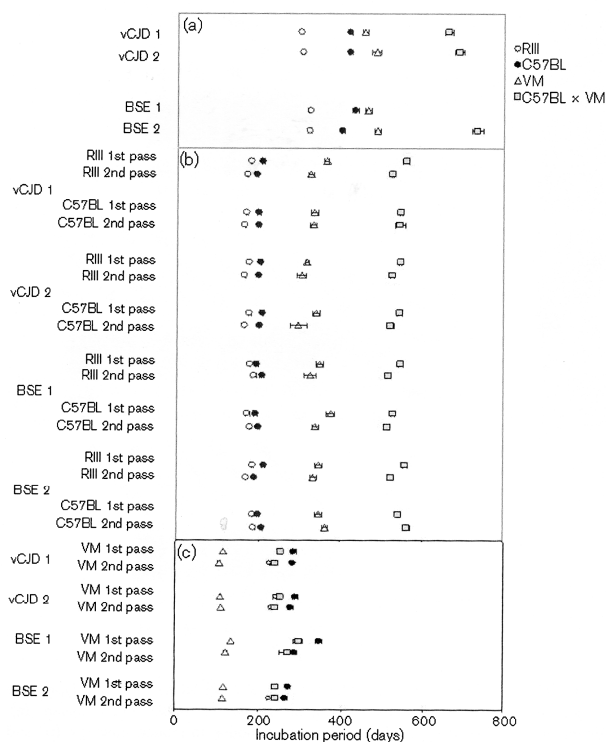


Fig. 5. (a) Mean (\pm SEM) incubation periods in wild-type mice after primary passage of vCJD and BSE. (b, c) Mean (\pm SEM) incubation periods from the first and second mouse passage of vCJD 1, vCJD 2, BSE 1 and BSE 2 in *Prn-p^a* (b) and *Prn-p^b* (c) mice.

tissue, as has been shown previously (Fig. 1a, c; Bruce *et al.*, 2001). Lesion profiling produced a consistent pattern irrespective of whether the tissue used for transmission was from the CNS or the lymphoreticular system (Fig. 2b). PrP deposition within the brains of mice challenged with vCJD lymphoid tissue homogenates was virtually indistinguishable from those found in mice challenged with vCJD brain homogenates (Fig. 3e, f). Transmission of vCJD from tonsil and spleen tissue into VM and RIII mice resulted in glycoform patterns closely resembling those found following transmission from vCJD brain tissue (Fig. 4c); however, the single RIII mouse inoculated with vCJD spleen tissue that was available for analysis had a substantially modified glycoform ratio (Fig. 4c). The apparent correlation between mouse *Prn-p* genotype and the relative abundance of the upper and lower bands comprising the unglycosylated doublet did not hold for transmissions from peripheral tissues.

DISCUSSION

In this paper, we confirm the original observations of Bruce *et al.* (1997) and extend them in a number of

important respects. Firstly, we have increased the numbers of transmitted cases to a total of 10, providing complete incubation period and lesion profile data in a larger panel of mouse strains. Secondly, we have extended the pathological description to include the biochemical and immunohistochemical analysis of abnormal PrP accumulation in the mice. Thirdly, we have analysed the transmission characteristics of the isolates after a further two passages in mice and finally, we have analysed transmissions from vCJD tissues other than tissues from the brain.

We consistently observed shorter incubation periods with inocula prepared from the cerebellar cortex compared with inocula from the frontal cortex. Although sampled from different vCJD cases, this suggests that titres of infectivity are higher in the cerebellar cortex than in the frontal cortex. Pathological changes in these two brain regions in vCJD patients are qualitatively similar, but accumulation of PrP is often more pronounced in the cerebellum with more frequent florid plaques (Ironside *et al.*, 2000), perhaps reflecting the apparent relatively high titre.

The difference in incubation period observed between the RIII and C57BL mice (both *Prn-p^a*), which is characteristic

of the BSE agent, was replicated in all seven transmissions from vCJD cases in which both mouse strains were included. In addition, subpassage of the vCJD agent in *Prn-p^a* and *Prn-p^b* mice resulted in the isolation of two distinct mouse-passaged strains, closely similar to the 301V and 301C strains which are derived by serial passage of BSE in VM and C57BL mice, respectively (Bruce *et al.*, 2002). Subpassage of vCJD through VM mice resulted in incubation periods of around 110 days in VM recipients, one of the shortest incubation periods for a mouse-passaged TSE strain in non-transgenic mice, mirroring the behaviour of the BSE agent in the same mouse strain (Bruce *et al.*, 2002). Interestingly, the biological properties of these two subpassaged vCJD-related strains were not reflected in differences in the PrP^{res} type found in the brains of the mice replicating these two strains of agent. Although no obvious changes were found in the glycosylation pattern, certain differences were observed in the unglycosylated fragment, which was seen as a doublet. The upper and lower bands of this doublet appeared to be associated with the different *Prn-p* genotypes in that the lower band was predominant in the *Prn-p^a* whereas the upper band predominated in *Prn-p^b* mice on primary transmission. These apparent differences in the biochemical profile of PrP^{res} might be attributed to differences in the polymorphic residues between *Prn-p^a* and *Prn-p^b* that result in different conformation or aggregation states of the misfolded form of the protein. However, the relationship did not hold when the subpassages were analysed and so the biochemical basis of this phenomenon and its biological significance remain unclear. Nevertheless, it resembles a phenomenon previously reported to occur in the BSE-derived 301V and 301C strains (Somerville *et al.*, 2005).

Previous transmission experiments have shown that titres of infectivity in vCJD spleen and tonsil are lower than in brain, resulting in extended incubation periods (Bruce *et al.*, 2001). In this study, we have found that although incubation periods are often longer in transmissions from lymphoid tissue, lesion profiles and patterns of PrP accumulation in the recipient mice are very similar to those found in vCJD brain transmissions (Figs 2 and 3), confirming previous findings that the strain properties of the agent are not substantially altered by the tissue in which it replicates (Beringue *et al.*, 2008). This is interesting because minor differences in PrP^{res} glycosylation and fragment size have been described in vCJD lymphoreticular tissues compared with the brain (Head *et al.*, 2004). These alterations in glycoform pattern are not conserved on transmission to mice, implying that the differences seen in PrP^{res} type between brain and lymphoreticular tissues in vCJD patients are tissue-specific epiphenomena and not relevant to the fundamental properties of the agent.

Bioassay in mice remains the definitive method for strain typing of prion disease isolates, but such experiments are often long and expensive. A number of new biochemical

approaches to strain typing in prion diseases have been described (Caughey *et al.*, 1998; Thomzig *et al.*, 2004; Spassov *et al.*, 2006). It will be interesting to see whether the biological strain typing properties of vCJD described in this study can be reproduced by these biochemical approaches.

In conclusion, in this extensive series of vCJD transmissions to wild-type mice, we confirm, using incubation period, lesion profile and glycoform analysis, that a BSE-related strain of agent is involved in vCJD, irrespective of the patient, the brain region or tissue type analysed.

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